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UNIVERSITÀ
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DBS
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Identification and functional analysis of AGP genes related to pollen tube guidance into the embryo sac in *Arabidopsis thaliana*

Ana Marta Brandão de Almeida Cardoso Pereira

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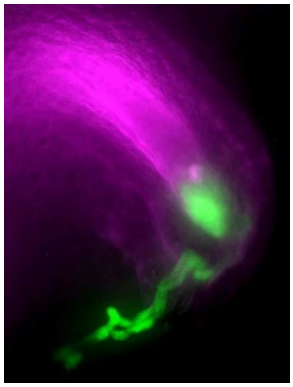
Joint supervision between Faculdade de Ciências da Universidade do Porto and Dipartimento di Bioscienze, Universitá degli Studi di Milano



Identification and functional analysis of AGP genes related to pollen tube guidance into the embryo sac in *Arabidopsis thaliana*

Ana Marta Brandão de Almeida Cardoso Pereira

PhD in Biology

Biology Department

2015

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FOREWORD

According to the number 7 of Article 6 from the Regulation of the Doctoral Program in Biology, Faculdade de Ciências da Universidade do Porto (and in agreement with the Portuguese Law Decree nº 74/2006), this thesis integrates the articles listed below, written in collaboration with co-authors. The candidate hereby declares that she contributed to conceiving the ideas, compiling and producing the data and analyzing the data, and also declares that she led the writing of all Chapters.

LIST OF PAPERS:

Chapter 1 - Pereira AM, Pereira, LG, Coimbra S. 2015. Arabinogalactan proteins: rising attention from plant biologists. Plant Reprod. 28: 1-15.

Chapter 2 - Costa M, Pereira AM, Rudall PJ, Coimbra S. 2013. Immunolocalization of arabinogalactan proteins (AGPs) in reproductive structures of an early-divergent angiosperm, *Trithuria* (Hydatellaceae). Annals of Botany. 111(2):183-190.

Chapter 3 - Pereira AM, Masiero S, Nobre MS, Costa ML, Solís M-T, Testillano PS, Sprunck S, Coimbra S. 2014. Differential expression patterns of Arabinogalactan Proteins in *Arabidopsis thaliana* reproductive tissues. Journal of Experimental Botany. 65(18):5459–5471.

Chapter 4 - Pereira AM, Masiero S, Nobre SM, Lopes AL, Mendes MA, Pinto SC, Costa ML, Coimbra S. 2015. “Love is strong, and you’re so sweet”: *JAGGER* is essential for synergid degeneration and polytubey block in *Arabidopsis thaliana*. Plant Cell. Submitted.

to my parents.

Carl Sagan once said *"every kid starts out as a natural-born scientist, and then we beat it out of them. A few trickle through the system with their wonder and enthusiasm for science intact."* You are the perfect family, I'm still a kid every day in the lab (and outside).

I am among those who think that ***science*** *has great* ***beauty****. A* ***scientist*** *in his laboratory is not a mere technician: he* ***is also a child confronting natural phenomena that impress him as though they were fairy tales.***

Marie Curie

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Hour for them or “Boa Mike!”. Thank you for helping me so much and for being such a good student. The three of us, together, we are the super girls! Never forget it!

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for that day in the *Alicantina* esplanade, when you almost forced me to go to IBMC to talk to Sílvia and ask her to be a volunteer in her lab, it all started there. Mostly and always, for the friendship, may the Super-Gualter always be with us and we never give up!

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ABSTRACT

The evolution of sexual reproduction is the core of flowering plants success in our planet. One of the main outstanding questions about how fertilization takes place is to show how the pollen tube is recruited and its growth supported into the female gametophyte. Though much research has been dedicated to pollen tube growth and fertilization, only some specific molecules have been shown to function as PT growth enhancers such as GABA in Arabidopsis and chemocyanin in *Lilium longiflorum*. To date, we still know very little of the intricate molecular network that controls pollen-pistil interactions and finally leads to the production of seeds in the model species *Arabidopsis thaliana*. Throughout the distinctive sporophytic tissues that comprise the pathway followed by a pollen tube until it reaches the embryo sac, the presence of Arabinogalactan Proteins (AGPs) is remarkable, as it was detected by immunolocalization studies in several plant species. The main goal of this thesis was to seek further insights about the localization and function of specific AGPs along these tissues in Arabidopsis, based on the central hypothesis that AGPs are essential for pollen tube guidance, attraction and reception into the embryo sac. AGPs belong to the family of Hydroxyproline-Rich Glycoproteins (HRPGs), being highly glycosylated, and ubiquitous in land plants. These proteins have long been proposed to have important roles in distinct phases of the sexual plant reproductive process, due, not only to their differential patterns of spatial and temporal localization in the reproductive tissues, but also owing to their very peculiar molecular characteristics.

Initially a first study using antibodies specific for AGPs sugar epitopes was made in a basal Angisoperm, *Trithuria submersa.* This work showed that AGPs could play a significant role in Trithuria reproduction, due to their specific presence in the pollen tube pathway. As a first approach to identify the AGPs involved in pollen tube attraction in the model species *A. thaliana*, we selected AGPs predicted to be specifically present in the pistil tissues, according to microarray data: AGP1, AGP9, AGP12, AGP15 and AGP23. Promoter analysis using multiple GFP fusions to a nuclear localization signal (NLS), GUS fusions, and *in situ* hybridization were the approaches utilized to confirm the AGP expression patterns. Promoter analysis allowed the detection of a specific and differential presence of these proteins along the pathway followed by the pollen tube during its journey to reach the egg and the central cell inside the embryo sac. *AGP1* is expressed in the stigma, the style, the transmitting tract, and in the chalazal and funiculus tissues of the ovules. *AGP9* is present along the vasculature of the reproductive tissues and *AGP12* is

expressed in the stigmatic cells, the chalazal and funiculus cells of the ovules, and the ovary septum. *AGP15* is expressed in all pistil tissues, except in the transmitting tract, while *AGP23* is pollen grain and pollen tube specific.

Lately, as a second step, T-DNA insertion mutants of AGPs were analysed for the ones with available knock-out mutants: AGP1, AGP10, and AGP20. *agp1*, *agp10* and *agp20* studies showed no visible phenotype. This was most probably due to the high probability of redundancy occurrence in this family of proteins. Concurrently, new microarray data became available regarding the expression of AGPs in the female gametophytic cells – synergid cells, egg and central cell. Based on these data a new AGP was selected for further analysis: AGP4, found to be highly expressed in the synergids, the source of the molecular pollen tube attractants into the embryo sac. *AGP4* showed expression in the style and the transmitting tract cells, in the integument cells of the ovule, near the micropyle, and in the synergids. *agp4* knock-out plants revealed a normal seed set. Remarkably, when cross pollinations between *agp4* and wild-type plants were carried out, we found multiple pollen tubes growing towards the same embryo sac, the so called polytubey phenotype. This phenomenon is highly frequent, occurring in at least 14 to 18% of the embryo sacs in every pistil pollinated with wild-type pollen. The reciprocal crosses were always performed and have shown the occurrence of about 1,7% of polytubey cases in the wild-type pistils when pollinated with *agp4* pollen. This led us to rename this gene after the rock'n'roll god, Mick Jagger, who "can't get no satisfaction", as JAGGER. After a detailed morphological study of *jagger* embryo sacs, we concluded that all the developmental process occurred normally. The only difference between this mutant and the wild-type flowers relied on the survival of the second synergid, which did not degenerate after zygote formation and embryo development in the mutant lines. The phenotypic studies led us to hypothesize the involvement of AGP4 in the blockage of polytubey by interfering with the consequent blocking of pollen tube attractants production.

With the aim to identify interaction partners of AGP4, an Arabidopsis cDNA library was screened using the yeast two-hybrid approach. Several putative interactors were found, most of them related to ABA signalling and stress or wound response, taking us back to older hypothesis that the invasion of the pistil tissues resembles host-parasite interactions.

This work reveals the expression pattern of several AGPs, providing new evidences for the detection of a subset of specific AGPs, being of significance for this field of study

and reinforcing the role of AGPs as prominent candidates for male-female communication during reproduction. This work also shows that AGP4 is essential to block polyspermy in Arabidopsis, probably by priming the pollen tubes to be received by the synergid cells and block the arrival of more pollen tubes or by controlling synergid cell death.

Keywords: pollen tube, guidance, pistil, embryo sac, double fertilization

RESUMO

A evolução da reprodução sexuada foi fundamental para o sucesso evolutivo das Angiospérmicas no nosso planeta. Uma das questões mais proeminentes sobre os mecanismos de fecundação relaciona-se com a determinação da forma como o tubo polínico é recrutado e o seu crescimento sustentado até ao gametófito feminino. Apesar de diversos estudos sugerirem o envolvimento de várias moléculas, tais como o GABA em Arabidopsis ou a quimiocianina em *Lilium longiflorum*, até à data, o mecanismo exato que conduz o tubo polínico até ao saco embrionário, é ainda desconhecido. As Proteínas Arabinogalactânicas (AGPs) têm sido detetadas através de estudos de imunolocalização nos diferentes tecidos esporofíticos que constituem o caminho seguido pelo tubo polínico até atingir o saco embrionário, em diferentes espécies de plantas. O principal objetivo desta tese foi investigar a localização e a possível função de AGPs específicas, ao longo destes tecidos em *Arabidopsis thaliana*, baseando-nos na hipótese central de que as AGPs são fundamentais para a recepção, direccionamento e atracção do tubo polínico para o saco embrionário. As AGPs pertencem a uma grande família de Glicoproteínas ricas em Hidroxiprolinas (HRPGs, *Hydroxyproline-rich Glycoroteins*) altamente glicosiladas e ubíquas nas plantas terrestres. Estas proteínas têm sido sucessivamente envolvidas nas diferentes fases do desenvolvimento reprodutivo das plantas, devido, não só, aos seus padrões temporais e espaciais de expressão nos tecidos reprodutivos, mas também devido às suas características moleculares muito específicas.

Um estudo inicial foi efetuado utilizando anticorpos específicos para o reconhecimento de epítopos de açúcares de AGPs numa Angisopérmica basal, a *Trithuria submersa*. Este trabalho demonstrou que as AGPs podem ter funções importantes na reprodução em Trithuria, tendo em conta os resultados obtidos relativamente à sua presença específica ao longo do caminho seguido pelo tubo polínico até à fertilização. Como primeira abordagem para a identificação de AGPs relacionadas com o crescimento do tubo polínico na espécie modelo *A. thaliana*, seleccionaram-se as AGPs com uma presença específica nos tecidos do pistilo, de acordo com resultados de microarrays: AGP1, AGP9, AGP12, AGP15, e AGP23. De forma a confirmar os padrões de expressão destes genes efetuou-se a análise da atividade dos respetivos promotores utilizando-se fusões de múltiplas proteínas de fluorescência verde com um sinal de localização nuclear, fusões com β-glucuronidase e hibridização *in situ*. A análise da expressão dos diferentes promotores permitiu a deteção da presença diferencial e específica destas proteínas ao longo do caminho seguido pelo tubo polínico até atingir a

oosfera e a célula central no interior do saco embrionário. A expressão da *AGP1* foi encontrada no estigma, estilete, tecido de transmissão e nos tecidos da caláza e do funículo dos óvulos. O gene da AGP9 revelou ser expresso ao longo dos tecidos condutores presentes nos tecidos reprodutivos, e o gene da AGP12 é expresso nas células estigmáticas, nas células da caláza e do funículo dos óvulos, e também no septo do ovário. Por sua vez o gene da AGP15 é expresso em todos os tecidos do pistilo, com exceção do tecido de transmissão, sendo o gene da AGP23 expresso especificamente no grão de pólen e tubo polínico.

Simultaneamente foram analisados mutantes de inserção de T-DNA nulos disponíveis para a AGP1, AGP10 e AGP20. Os estudos dos mutantes *agp1*, *agp10* e *agp20* não revelaram qualquer fenótipo visível. Possivelmente devido à elevada probabilidade de ocorrência de redundância genética nesta grande família de proteínas. Concomitantemente, foram disponibilizados novos dados de *microarray* relativos à expressão das AGPs nas células do gametófito feminino – sinergídeas, oosfera e célula central. Com base nestes dados uma nova AGP foi selecionadas para análise: a AGP4, com níveis de expressão génica elevados nas sinergídeas, a principal fonte de moléculas de atracão para o crescimento do tubo polínico até ao saco embrionário na sua última fase de direcionamento.

A *AGP4* é expressa nas células estigmáticas, nas células do tecido de transmissão, nos tegumentos do óvulo junto à região micropilar e nas sinergídeas. Plantas com expressão nula da *AGP4*, plantas *agp4*, apresentaram uma produção de sementes normal. Notavelmente, quando se realizaram polinizações cruzadas entre plantas *agp4* e plantas da variedade selvagem, foram observados vários casos em que dois tubos polínicos cresceram em direção a um mesmo saco embrionário, um fenótipo atualmente designado por *polytubey*. Este fenótipo revelou-se bastante frequente, ocorrendo em cerca de 14 a 18% dos sacos embrionários de cada pistilo observado. O cruzamento recíproco mostra a ocorrência de apenas 1,7% de situações de *polytubey*. Dado o fenótipo observado decidimos chamar a esta AGP4, JAGGER, em homenagem ao deus do rock'n'roll Mick Jagger. Após estudo detalhado, a nível morfológico, dos sacos embrionários deste mutante verificamos que o seu desenvolvimento decorre normalmente, como na planta selvagem. A única diferença observada entre as flores do *jagger* e da planta selvagem foi a presença da segunda sinergídeas, que não degenera após formação do zigoto ou desenvolvimento do embrião, nas linhas mutantes. Assim sendo, a análise de mutantes *agp4* conduziu-nos à hipótese do envolvimento da AGP4

no bloqueio do crescimento de mais do que um tubo polínico para um mesmo saco embrionário, e consequentemente, interferindo com o bloqueio da produção de moléculas de atracção pelas sinergídeas, bem como com a morte da segunda sinergídea.

De modo a identificarem-se possíveis moléculas que possam interagir com a AGP4, efetuou-se um rastreio a uma biblioteca de cDNA de Arabidopsis através do método de *Yeast Two Hybrid*. Várias moléculas foram identificadas como possíveis moléculas de interação com a AGP4, a maioria relacionada com as vias de sinalização do ácido abcísico (ABA) e com respostas de stress biótico e abiótico. Isto leva-nos de volta à já antiga hipótese de que a invasão dos tecidos do pistilo pelos tubos polínicos se assemelha, em parte, às interações hospedeiro-parasita.

Este trabalho revela finalmente o padrão de expressão de várias AGPs específicas ao longo dos diferentes tecidos reprodutivos, reforçando a importância das AGPs como moléculas essenciais para a comunicação entre o gametófito masculino e feminino durante a reprodução das plantas com flor. Este trabalho demonstra também que a AGP4 é fundamental para o bloqueio da polispermia em Arabidopsis, provavelmente através da preparação dos tubos polínicos para serem devidamente reconhecidos pelas sinergídeas, bloqueando a entrada de mais do que um tubo polínico, ou através do controlo da morte celular das sinergídeas.

Palavras-chave: tubo polínico, direcionamento, pistilo, saco embrionário, dupla fecundação

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Chapter 3 - Differential expression patterns of Arabinogalactan Proteins in Arabidopsis thaliana reproductive tissues

Appendix 1 – Supplemental material from Chapter 3

Appendix 1 – Supplemental material from Chapter 4

LIST OF ABREVIATIONS

µm	Micrometer
AG	Arabinogalactan
bp	Base pair
Ca^{2+}	Calcium ion
CC	Central cell
cDNA	Complementary DNA
CLSM	Confocal Laser Scanning Microscope
Cys-rich	Cysteine-rich
DAPI	4',6-diamidino-2-phenylindole
DIC	Differential Interference Contrast
DIG	Digoxigenin
DNA	Desoxiribonucleic acid
DNase	Desoxirribonuclease
dSY	Degenerated synergid
EC	Egg cell
ECM	Extracellular Matrix
FG	Female Gametophyte
FISH	Fluorescence *In Situ* Hybridization
FITC	Fluorescein isothiocyanate
FLA	Fasciclin-like AGP
FM	Functional Megaspore
GABA	γ-aminobutyric acid
gDNA	Genomic DNA
GFP	Green Fluorescente Protein
GPI	Glycosylphosphatidylinositol
GUS	Beta-glucuronidase
h	hour
HRGPs	Hydroxyproline Rich Glycoprotein
kDa	Kilodalton
Lys-rich	Lysine-rich
M	Molar
min	minutes
mm	milimeter

mAb	Monoclonal antibody
mg	Milligram
MG	Male Gametophyte
Min	Minute
mL	Mililiter
mM	Milimolar
MMC	Megaspore Mother Cell
NJ	Neighbour Joining
NLS	Nuclear Localization Signal
nm	Nanometer
oligodT	Deoxy-thymine nucleotides
ON	Overnight
PAT	Phosphinotricin-Acetyltransferase
PBS	Phosphate Buffer Saline
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PELPIII	Pistil-specific Extensin-Like Protein
PG	Pollen Grain
PI4P	Phosphatidylinositol-4-phosphate
PMC	Pollen Mother Cell
PT	Pollen Tube
qPCR	Quantitative PCR
RLKs	RLKs
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Room Temperature
RT-PCR	Reverse-transcrption PCR
RU	Reproductive Unit
RUB1	Related to ubiquitin 1
SDS	Sodium Dodecyl Sulphate
SFA	Synergid Filiform Apparatus
SP	Septum
SSC	Saline-Sodium Citrate buffer
ST	Style
SUFP	Sirius Ultramarine Fluorescente Protein

SY	Synergid
T-DNA	Transfer DNA
TT	Transmitting Tract
TTS	Transmitting Tract Specific
UTR	Untranslated Region
Wt	Wild-type
Y2H	Yeast Two Hybrid

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CHAPTER 1

GENERAL INTRODUCTION

This chapter was partially based on:

Pereira AM, Pereira LG, Coimbra S. 2015. Arabinogalactan Proteins: rising attention from plant biologists. Sex Reprod. Plant Reprod. 28: 1-15.

GENERAL INTRODUCTION

"By 2050 we will need to feed two billion more people. How can we do that without overwhelming the planet?" (Foley, 2011, 2014). This is a fundamental question presently, which is still ignored by most of our politics and people in general. But, it is a reality. The demand for more crops to feed people is the main reason experts say we will need to double crop production by 2050. Yet, wild pollinators are dying, 75 billion tons of soil disappear every year, and droughts are becoming more common. Until today, an increasing world area have been used to produce more food, to feed people and livestock, and to produce biofuels. Agriculture has led to the loss of countless ecosystems, including North America prairies, the Atlantic forest of Brazil, and many tropical forests that continue to be cleared at shocking rates. To continue the transformation of forests into agricultural fields it is not an option anymore. It would only destroy a big part of our biodiversity and unbalance the planet ecosystems (Fig. 1).

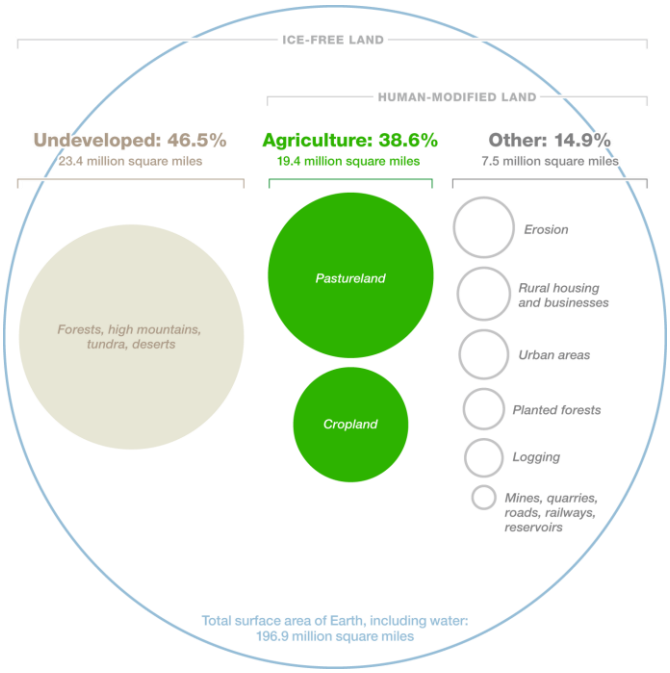

Figure 1 – Schematic representation of the occupation of the total surface area of the Earth. (Adapted from National Geograpgic, 2014).

The demand to increase cereals production, both for human and animal feed, and also for the production of biofuels, depending mainly on energy prices and government

policies, will grow faster than expected. A balance is needed between producing more food and nourishing our planet for our future generations. A big shift in thinking is needed in the main world leaders and population, which is not going to be easy. The solution to this problem will necessarily depend on increasing crop yield per unit area, rather than extending the area of arable land (FAO, 2014).

In order to improve crop yields, that feeds nearly half the world, it is fundamental to our countries to greatly increase the investments in agricultural and plant sciences research. Agricultural research represents now 5% of the total research and development funding worldwide (Pardey and Pingali). Plant breeding to get better crops was always a useful technique for any producer, but a very slow process. Nowadays, with all the new technologies available by genetic engineering, this can be easily overcome. Although, still some important mind changes are needed in order for the world to better accept and use genetically modified crops (Grierson *et al.* 2011; McGloughlin 2010).

With grains and fruits comprising over a ninety percent of the world's agricultural production (FAO, 2014), it can safely be said that double fertilization in flowering plants is essential for human life. In the last two decades significant advances have been achieved regarding the understanding of the mechanisms involved in sexual plant reproduction. A deeper understanding of how seeds are formed and how all this process is controlled at the molecular level is of paramount importance to further support the development of ground-breaking systems to improve crop yield. As applied sciences and/or technology will never reveal its usefulness based on anything else, but **blue sky research**, or **fundamental research**, this kind of studies are vital.

This thesis aims to get further insights about the involvement of the cell wall Arabinogalactan Proteins (AGPs) in the sexual plant reproductive processes. This Chapter revises the main topics regarding the different steps of sexual plant reproduction and it also describes the knowledge acquired until the present time about AGPs association with plant reproduction.

Scope of the thesis: identification and functional analysis of AGP genes related to pollen tube guidance into the embryo sac in *Arabidopsis thaliana*

In Angiosperms sexual reproduction encompasses complex mechanisms responsible for a unique fertilization process: double fertilization, which involves the formation of two sperm cells and two female gametes, the egg cell and the central cell, leading to two fertilization events that will initiate seed development (reviewed in Beale and Johnson, 2013; Dresselhaus and Franklin-Tong, 2013; Palanivelu and Tsukamoto, 2011). This implies complex interactions between male gametophyte, sporophyte and female gametophyte. The female gametophyte is the embryo sac, a structure embedded in the ovule tissue, comprised of seven cells: three antipodal cells at the chalazal pole, one central cell with two polar nuclei, two synergids and the egg cell at the micropylar pole. The male gametophyte is a three-celled structure comprised of two sperm cells encased within a vegetative cell (Higashiyama and Hamamura, 2008).

Fertilization begins with pollen hydration in the stigma, and posterior germination producing a pollen tube. Successful fertilization depends on the precise guidance of the pollen tube throughout the different pistil tissues into the embryo sac. In the initial sporophytic phase of attraction, the pollen tube grows inside the highly specialized pistil tissues of the style and the transmitting tract extracellular matrix extremely enriched in glycoproteins, polysaccharides and glycolipids (Crawford and Yanofsky, 2008), until it reaches the funiculus. During the gametophytic phase, the pollen tube grows from the funiculus until the micropylar end of the embryo sac (Higashiyama *et al.* 2003; Higashiyama and Hamamura, 2008). In the micropyle, the pollen tube enters through the filiform apparatus into one synergid, ceases growth, ruptures and releases its two sperm cells. These cells nuclei migrate to the egg and central cell, and fuse accomplishing double fertilization (Lord and Russell, 2002; Punwani and Drews, 2008).

Arabinogalactan Proteins (AGPs) are a class of highly glycosylated hydroxyproline-rich proteins ubiquitous in plants, abundant in cell walls, plasma membranes and extracellular secretions (Majewska-Sawka and Nothnagel, 2000). Most AGPs are predicted to be glycosylphosphatidylinositol (GPI) anchored to the plasma membrane (Borner *et al.* 2002). The presence of GPI-anchors provides mechanisms by which AGPs could be involved in signalling processes (Gaspar *et al.* 2001). This fact together with the

presence of AGPs in female tissues of *A. thaliana* and other species, and with a very specific pattern of distribution (Coimbra and Salema, 1997; Wu *et al.* 2001; Coimbra and Duarte 2003; Coimbra *et al.* 2007), makes them good candidates as important players in the complex interactions between male gametophyte, sporophyte and female gametophyte.

In this thesis we focus specifically in identifying these AGPs and to analyse their precise function in female tissues. Several members of the active signalling cascade during pollen tube growth and reception have been identified (Dresselhaus and Franklin-Tong, 2013; Bleckmann *et al.* 2014), but the AGP role(s) remains unknown. To obtain further insights into the molecular mechanisms regulating pollen tube guidance, three different questions were addressed in this PhD thesis and different experimental approaches were conducted to answer them:

- Is the presence of AGPs along the reproductive tissues conserved in basal Angiosperms?
- Which specific AGPs are present along the reproductive tissues of the model plant *Arabidopsis thaliana*?
- Which are the probable functions of these, previously identified, AGPs?

To address the first question, we used Trithuria, the sole genus of Hydatellaceae, a family of the early-divergent angiosperm lineage Nymphaeales (water-lilies). We used several monoclonal antibodies to identify different AGP epitopes in *T. submersa* in order to better understand the diversity of these proteins and their functions in flowering plants. Immunolabelling of different AGPs and pectins was performed in reproductive structures of *T. submersa* at the stage of early seed development, by immunofluorescence of specific antibodies (see Chapter 2).

To identify specific AGPs expressed in the pathway followed by the pollen tube in *A. thaliana* pistils tissues, several techniques were used, such as: *in silico* analysis, promoter fusion studies, *in situ* hybridization and quantitative RT-PCR techniques. The individual distribution of specific Arabidopsis AGPs: AGP1, AGP9, AGP12, AGP15 and AGP23, throughout reproductive tissues was described and possible roles were pointed out for them in several reproductive processes (see Chapter 3).

Along with these results, we provided evidence that AGPs are important components of the sexual plant reproductive process and a new role for AGP4 is proposed in the mechanism of multiple pollen tube block in Arabidopsis (see Chapter 4).

This thesis comprises five chapters, including this introductory Chapter 1 and a final Chapter 5, with some concluding remarks and future perspectives in this field of study.

In this thesis, published work is used and is preceded by a short note indicating Ana Marta Pereira's contribution to the article if she is not first author. Importantly, every chapter – even if it is not published (yet) – is written in the form of a publication including introduction, results, discussion, material and methods and references. Therefore, references might be cited more than once and materials and methods are only specified within the chapters. Supplemental Information to Chapter 3 and 4 can be found in the Appendixes 1 and 2, respectively.

Double fertilization – twosomes, a love story

Double fertilization is a key process for successful development of a seed and the establishment of a new plant generation. It is a unique process, characteristic of Angiosperms, where two fertilizations occur simultaneously. This process was first described by S. Nawaschin in *Lilium martagon* and *Fritillaria tenella* in 1898. L. Guinard has confirmed the same phenomenon one year later independently in *Lilium martagon* e *Lilium pyrenaicum* (Raghavan, 2003).

In flowering plants, the male gametophyte (MG) develops inside the anthers locules (Fig. 2A), part of the stamen, the male reproductive organ. It is released as the mature pollen grain (PG) containing a vegetative cell that will develop into a pollen tube (PT), and a generative cell that will generate by mitosis two sperm cells: the male gametes (McCormick, 2004; Borg and Twell, 2010).

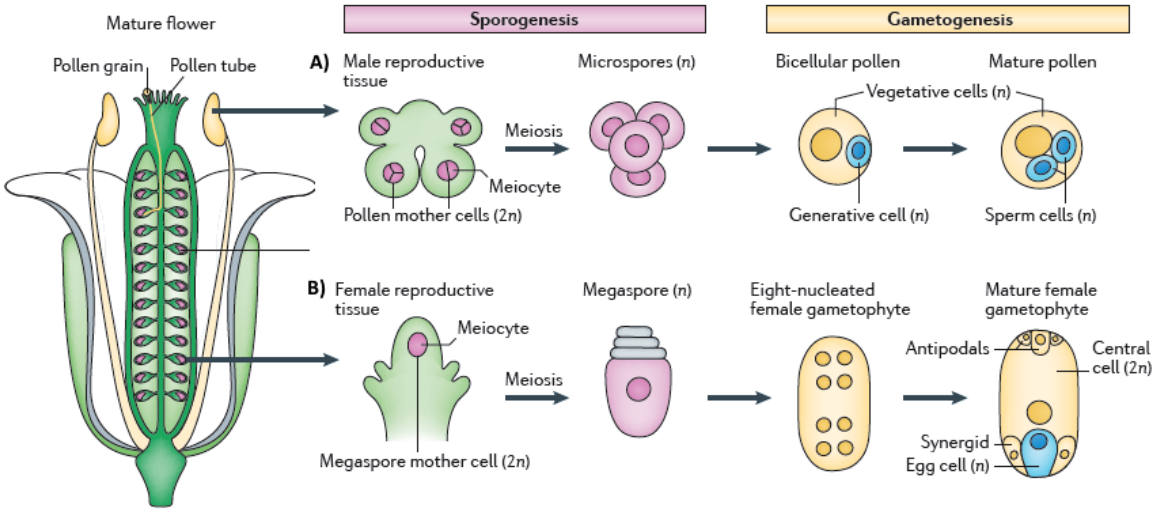

Figure 2 – Mature flower showing in detail male and female gametophyte's formation.

A. The pollen mother cells, located inside the anther locules, undergo meiosis generating the haploid microspores in a process called sporogenesis. Sporogenesis is followed by gametogenesis, where a mature pollen is formed, containing a vegetative cell engulfing two sperm cells, the male gametes. **B.** The megaspore mother cells embedded in the ovular nucellar tissues also undergoes meiosis generating four haploid megaspores. From these, only one will survive and become the functional megaspore, which, after three rounds of mitosis and cellularization will give rise to the mature female gametophyte, the embryo sac. (Adapted from Kawashima and Berger, 2014).

The female gametophyte (FG) develops inside the ovule, deeply embedded in the sporophytic tissues of the pistil (Fig. 2B). During megasporogenesis a cell from the

nucellus, the archesporial cell, differentiates into the megaspore mother cell (MMC), which undergoes meiosis giving rise to four haploid megaspores. One of these megaspores develops into the functional megaspore and the remaining three megaspores will undergo programmed cell death. In the *Polygonum*-type of female gametophyte (Yadegari and Drews, 2004), the functional megaspore undertakes three rounds of nuclear divisions (mitosis without cytokinesis) resulting in a coenocyte with eight nuclei at distinct positions of the female gametophyte. After migration of the nuclei and cellularization, the mature embryo sac or FG is formed, a seven celled, eight-nucleated structure that comprises: three antipodal cells positioned in the chalazal pole of the ovule, one diploid central cell and two synergid cells surrounding the egg cell, at the micropylar pole of the ovule. Here are kept the two female gametes: the central cell and the egg cell (Huang and Russell, 1992; Drews *et al.* 1998; Yadegari and Drews, 2004).

Once the mature PG reaches the stigmatic cells and is recognized as compatible pollen, it adheres to the stigmatic cell and hydration begins, leading to the formation of a protrusion (Fig. 3A and 3B). This protrusion will become the pollen tube that is responsible for the carriage of the two sperm cells into the embryo sac. The fast and tightly controlled growth of the PT tip is ensured by a series of communication and consequent signalling cascades, which result from several interactions between the PG and the PT with the female sporophytic and gametophytic tissues. Given this, the PT tip grows along the extracellular matrix (ECM) of the stylar and transmitting tract (TT) tissues until it perceives signals that will make it turn abruptly in the direction of an ovule. At this moment the PT quickly turns into the placenta tissues, growing along the funiculus until it reaches the embryo sac entrance: the micropyle (Fig. 3A, highlighted by a dotted red square, Fig. 3C, for a more detailed view). Once in the micropylar region the PT enters the embryo sac through the filiform apparatus of one of the two synergids, ceasing its growth, rupturing and releasing its two sperm cells. The invaded synergid dies after PT entrance, and the persisting synergid will undergo programmed cell death after successful fertilization of both female gametes. The sperm cells nuclei migrate to the egg and central cell, and fuse with them, giving rise, respectively to the embryo and its nourishing tissue, the endosperm, finally accomplishing double fertilization (Fig. 3C) (reviewed in Beale and Johnson, 2013; Dresselhaus and Franklin-Tong, 2013; Palanivelu and Tsukamoto, 2011).

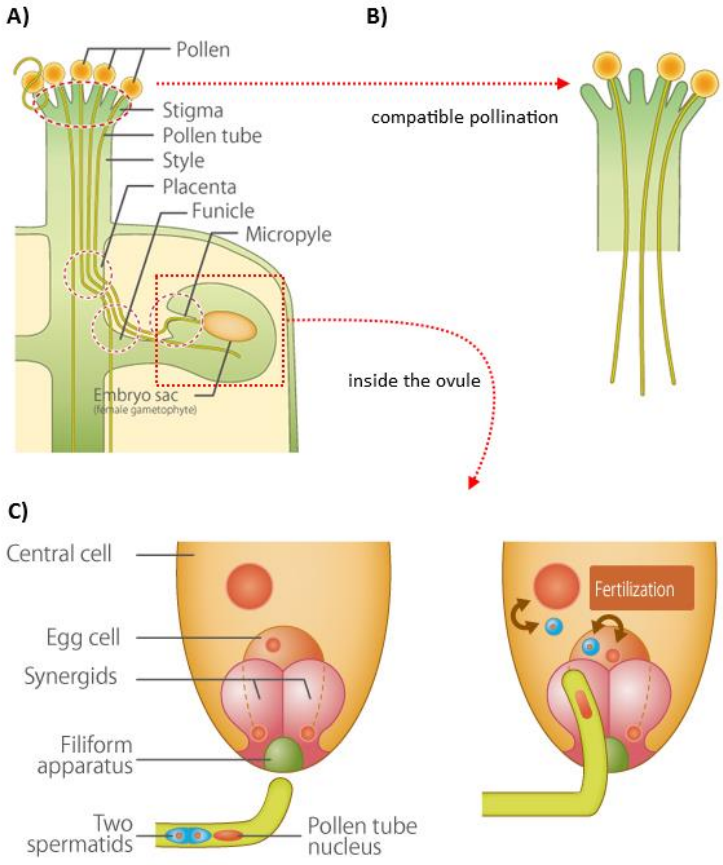

Figure 3 – Pollen tube growth and double fertilization.

A. The compatible pollen grains germinate in the stigma, forming the pollen tube that grows along the style tissues. The regions circled by red broken lines represent key points for pollen tube guidance, where specific signals are essential to regulate the PT growth from this points onwards. **B.** The PT elongates through the pistil tissues until turning in the direction of one ovule. **C.** Once the PT is in the micropyle, and after a series of signalling cascades, it will enter one of the synergids through the filiform apparatus region, releasing the two sperm cells nuclei, one will fuse with the central cell and the other one with the egg cell. (Adapted from CSLS/The University of Tokyo).

The following sections highlight the several communication levels between the male gametophyte and the female reproductive tissues: (i) interaction events during the growth of the PT throughout the female flower sporophytic tissues, (ii) molecular mechanisms during pollen tube reception, and (iii) double fertilization and multiple pollen tube blockage.

Pollen tube germination and growth – the love story begins

PT growth from the stigma until the embryo sac may be divided in two different phases:

- **a sporophytic phase**, that comprises PT growth from the stigma, through the style and the TT, and independent from molecular cues from the FG;
- **a gametophytic phase,** which depends on molecular cues provided by the FG, and refers to PT growth from the moment it makes a quick turn into the septum surface and enters the funiculus targeting the micropyle of the embryo sac.

It starts with a PG landing on the dry stigma of an Arabidopsis pistil. This PG must adhere to the stigmatic cells to hydrate and, if compatible, it will germinate producing the sperm cell carrier PT. The initial adhesion of the PG to the stigma surface is largely dependent on the outer membrane of the PG, the exine (Zinkl *et al.* 1999). Immediately after contact with stigmatic cells, the pollen coat is extruded of the exine wall against the stigma surface, forming a 'foot' that strongly sticks to the stigma (Chapman and Goring, 2010). Hydration of the PG follows this step, depending on the pollen coat lipids to control the movement of water from the stigma (Wolters-Arts *et al.* 1998; Mayfield and Preuss 2000; Mayfield *et al.* 2001). All this steps are described in detail in Figure 4.

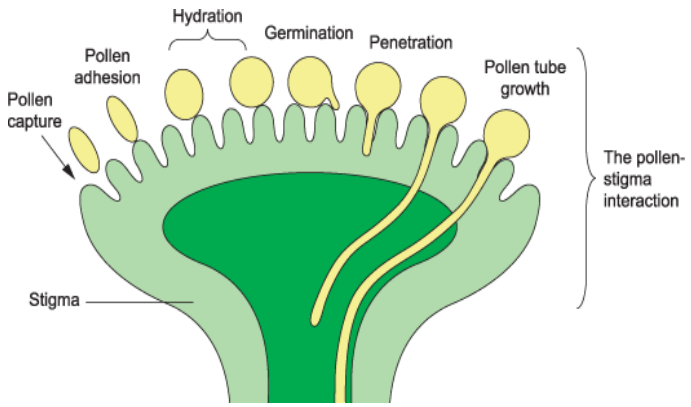

Figure 4 - Different stages of the pollen–stigma interactions.

Schematic representation of a typical stigma of the dry papillate type found in species from the Brassicaceae. Pollen is shown at various stages of development on the stigma: capture, adhesion, hydration, germination, penetration and PT growth into the stigma tissues. (Adapted from Hiscock and Allen, 2008).

Though the PG has great responsibility in initiating compatible PG germination in the stigma, its female partner is also essential for this successful interaction. Exo70A1, a exocyst complex subunit present in *A. thaliana* stigmas is needed for acceptance of compatible PG. Exo70A1 is proposed to act in the polarized secretion of stigmatic papillar resources to the pollen grain, to deliver vesicles containing aquaporins for increased water permeability, allowing pollen hydration, as well as cell wall–modifying enzymes, to allow PT penetration through the stigma (Samuel *et al.* 2009). Phosphatidylinositol-4-phosphate (PI4P) was shown to be essential for this step through the study of mutants with reduced levels of PI4P in the stigma. These mutants revealed slower rates of PG hydration and showed maternal fertility defects due to a higher level of failed pollinations (Chapman and Goring, 2011). This study suggested that phospoinositides, which are important lipids

involved in polarized secretion in general, have a specific role in pollen hydration. Monitoring of Ca^{2+} dynamics during pollination both in PG and stigmatic cells revealed some interesting results (Iwano *et al.* 2009). The cytosolic [Ca^{2+}] increased at different time points: in PG, after hydration until germination, and in the stigmatic cells after PG adhesion and hydration, after PT emergence from the PG and after the PT penetrated the stigmatic cell wall. This shows that Ca^{2+} acts most probably as an intracellular signalling factor during PG and stigmatic cells interaction, for PT formation and initial polarized tip growth.

Finally, after initiating the growing through the stigmatic cell walls, the PT will start a long journey that will lead it into the Arabidopsis embryo sac.

Pollen tube growth on the super-highway – courtship

Once germinated and the PT formed, a new phase in the PT-pistil interactions story starts. Successful fertilization depends on the precise guidance of the PT throughout the different pistil tissues into the embryo sac. In Arabidopsis PTs penetrate stigmatic cells and grow directly through the cell wall and into the cytosol, forcing their way through the basal wall region and into the TT (Hiscock and Allen, 2008). The PT has an intercellular growth through the stylar cells until reaching the TT (Hülskamp *et al*. 1995; Johnson and Preuss, 2002; Lord and Russell, 2002).

In the initial sporophytic phase of attraction, the PT grows through the TT, a specialized tissue extremely rich in glycoproteins, polysaccharides and glycolipids, which are thought to facilitate and nourish the PT development (Faure *et al.* 2002; Crawford and Yanofsky, 2008). In *Nicotiana tabacum* AGPs called TTS (Transmitting Tract Specific) are abundant in the extracellular matrix of the TT, being related to PT guiding into the ovules. These proteins stimulate PT growth *in vitro*, are able to attract PTs in *semi-in vivo* assays and are essential for optimal PT growth *in vivo*. They seem to function as a nutrient source and act as adhesion molecules for PT growth (Cheung *et al.* 1995; Wu *et al.* 2001). Arabidopsis *NTT* (*no transmitting tract*) mutants, with abnormal TT development, present severe defects in PT growth, which is slower, leading to a reduced fertilization percentage (Crawford *et al.* 2007). *NTT* encodes a C2H2/C2HC zinc finger transcription factor involved in ECM production and is essential for programmed cell death (PCD) in TT after pollination (Crawford *et al.* 2007). This shows NTT importance for the fast PT growth along this tissue.

POLLEN ON PISTIL 2 (POP2) encodes a γ-aminobutyric acid (GABA) transaminase involved in degrading GABA, establishing an increasing [GABA] gradient from the stigma until the ovule integuments, the PT growth pathway (Palanivelu *et al.* 2003). This gradient is believed to sustain PT guidance though the TT. A plant lipid transfer protein, SCA (stylar cysteine-rich adhesion) was shown to be secreted from the TT epidermis and involved in adhesion-mediated PT guidance by the formation of an adhesive pectin matrix that guides the PTs towards the ovules (Mollet *et al.* 2000; Park *et al.* 2000). In *Lilium longiflorum* a chemotropic compound from the stigma was identified as a chemocyanin, a small cell wall protein, which is able to reorient PT growth in *in vitro* assays and in the stigma, an activity enhanced by the cooperation with SCA (Kim *et al.* 2003). More recently, the amino acid D-serine was shown to play a role in PT guidance through the TT by mediating Ca^{2+} influx into the PT cytoplasm involving glutamate receptor–like channels (Michard *et al.* 2011).

Still, it is not clear how all these TT constituents interact with the growing PT promoting or/and supporting its growth. It is clear that there must be a tightly controlled activation and/or inactivation of specific receptors in PTs and TT cells as well as the production of ligands by the TT to coordinate the PT growth towards the embryo sac. Several microarray-based approaches have been used to identify novel intervenients in PT-sporophyte interaction mechanisms (Tung *et al.* 2005; Qin *et al.* 2009; Boavida *et al.* 2011). Interestingly, all the studies show enrichment in secreted proteins and cell-wall-related proteins potentially involved in extracellular signalling and ECM modifications. Besides the importance of these interactions for the PT growth along the pistil tissues, all the different components present in these tissues point out for a contact-mediated competence that must be conferred by the stigma and style to the PT in order for it to become receptive to ovule signals (Palanivelu and Preuss, 2006).

Funicular & micropylar growth – sex laws

Contrarily to the PT guidance phase described above, completely dependent on signals from the sporophyte, the funicular and micropylar growth of the PT depends only from cues delivered by the FG. The Arabidopsis mutants *magatama* (*maa1* and *maa3*, the latter encoding a helicase*)* show an abnormal FG development with PTs being targeted to the micropyle region, but not entering the synergids (Shimizu and Okada, 2000; Shimizu *et al.* 2008). Also in Arabidopsis, two predicted K^+ transporters CHX21 and CHX23 present in PTs, are essential for PT guidance. *chx21* and *chx23* PTs grow normally trough

the TT but fail to turn into the ovule direction and do not grow along the funiculus. The perception of some ovule signals that are critical to shifting the axis of PT polarity and directing its growth toward the ovule most probably fails in these mutants (Lu *et al.* 2011). Mitogen-activated protein kinases MPK3 and MPK4 from pollen were additionally shown to be essential for PT guidance during the funicular guidance phase, although the micropylar guidance was not affected (Guan *et al.* 2014). A most recent study revealed that phytosulfokine (PSK) is essential to guide the PT from the TT to the embryo sac. PSK is perceived by receptor kinases and requires sulfation by a tyrosylprotein sulfotransferase (TPST) to be active. *pskr1-3 pskr2-1* and *tpst* siliques present a high level of unfertilized ovules and loss of funicular guidance, suggesting the importance of PSK for funicular guidance (Stührwohldt *et al.* 2015). Curiously, PSKR1 was recently shown to be involved in plant defense to pathogens as well as PSK (Mosher and Kemmerling, 2013). The MPK3/MPK6 signalling pathway and the PSK signalling may link common signalling networks in plant stress response and pollen-pistil interaction. Clearly, PTs require a multiplicity of signalling pathways in order to perceive and repond to ovule signalling and determine its growth direction.

Considerable progress has been made in the last years in order to better understand what controls the last phase of PT guidance, the micropylar guidance phase. When PTs arrive at the micropylar region they must grow through this opening between the integuments of the ovules to reach the filiform apparatus, which are invaginations from the synergids' cell walls that greatly increase their extracellular contact area, as typical for active secreting cells (Huang and Russel, 1992). In *Torenia fournieri*, a species with a naked embryo sac, it was shown that the synergids are the FG cells responsible for the gametophytic guidance phase. Laser ablation studies revealed that both synergids are essential for PT guidance, but a single synergid is also capable of attracting PTs into the embryo sac (Higashiyama *et al.* 1998, 2001). Moreover, in Arabidopsis, the importance of the synergids as the source of guiding molecules in this phase was shown, by studying a transcription factor mutant, *myb98*. In this mutant the ovule develops normally, except for the synergids filiform apparatus, which does not develop normally, impairing PT guidance at this step (Kasahara *et al.* 2005; Punwani *et al.* 2008). So, the synergid cells are the main source for chemo-attractants necessary for micropylar PT guidance.

So far, molecular attractants produced by the synergid cells were identified in three different species. In *T. fournieri* a group of defensine like polypeptides (DEFL) were identified as PT attractants produced by the synergids and secreted to the filiform apparatus' surface, the LUREs (Okuda *et al.* 2009). A DEFL subgroup CRP810/*At*LURE1,

was also identified in Arabidopsis, revealing to be essential for micropylar PT guidance (Takeuchi and Higashiyama, 2012). In *Zea mays*, the EGG APPARATUS1 (*Zm*EA1), a small hydrophobic precursor protein of 94 aa, is present not only in the synergids, but also in the egg cell, in the filiform apparatus and in the micropylar nucellus, and it is required for micropylar PT guidance (Márton *et al.* 2005, 2012). The synergids are not the only cells regulating PT guidance, in *Arabidopsis*, *CENTRAL CELL GUIDANCE* (*CCG*) is expressed in the nucleus of the central cell and might function as a transcriptional regulator mediating PT guidance, since PTs fail to target the micropyle of mutant ovules (Chen *et al.* 2007). Furthermore, the egg cell expressed GEX3 protein was shown to play a role in ovular guidance of the PT (Alandete-Saez *et al.* 2008).

On the male side only two receptor-like kinases (RLKs) were shown to impair micropylar PT guidance, LOST IN POLLEN TUBE GUIDANCE1 (LIP1) and 2 (LIP2). They are both localized in the PT membrane and are involved in the AtLURE1-dependent guidance mechanism (Liu *et al*. 2013) although more evidences are necessary to prove that LIP1 and LIP2 interact with AtLUREs. According to Higashiyama and Hamamura (2008) it is still unclear if there is only one attractant molecule produced by the synergids to guide the PTs into the embryo sac or if several molecules work redundantly or even together to control such an important step in plant reproduction. It is more likely that several molecules involved in an intricate signalling pathway are acting in this micropylar guidance phase, since, until today, there is no known mutant where a total blockage of PT growth into the embryo sac is observed.

Pollen tube burst, sperm cell release and polytubey block: one life stand

Once in the ovule micropyle the PT must enter the embryo sac trough one of the synergids, arrest growth and burst inside releasing the two sperm cells. A recent study showed that the PT does not enter directly into the synergids trough the filiform apparatus but rather grows along this structure entering the embryo sac in a region with less cell wall invaginations (Leshem *et al.* 2013). In the recent years, the molecular interactions involved in PT reception by the synergids as well as between the sperm cells and the female gametes have been thoroughly studied shedding some light into all these mechanisms (reviewed in Bleckmann *et al.* 2014). Although there is a long way to go until

we comprehend all the mechanisms regulating such an important process for seed formation.

One of the first proteins to be related to PT reception by the synergids was FERONIA/SIRÉNE (FER/SRN), a receptor like serine/threonine kinase that localizes to the synergids filiform apparatus (Huck *et al.* 2003; Rotman *et al.* 2003; Escobar-Restrepo *et al.* 2007). *fer/srn* PTs fail to arrest growth, continue growing and do not release the sperm cells. This showed the active involvement of FER/SRN in mediating the signalling pathway responsible for PT-synergid interaction. FER, as a *Catharanthus roseus* RLK1-like kinase (CrRLK1Ls) possesses a malectin-like extracellular domain, thus, its ligand could be a carbohydrate or glycoprotein from the cell wall (Lindner *et al.* 2012a). The search for more candidate molecules involved in this step identified LORELEI (LRE) as a glycosylphosphatidylinositol (GPI)-anchored protein, predominantly expressed in the synergid cells. *lre* PTs present a similar phenotype to that of *fer*/*srn*, where the PT enters the embryo sac but fails to arrest and release the sperm cells (Capron *et al.* 2008). LRE is localized in the plasma membrane, therefore being a perfect candidate to participate in the PT-synergid interactions (Tsukamoto *et al.* 2010). Another gene identified whose loss-of-function resemble the *fer* phenotype is *NORTIA* (*NTA*), which encodes the *MILDEW RESISTANCE LOCUS O 7* (*MLO7*) gene (Kessler *et al.* 2010). NTA localizes to cellular uncharacterized compartments, and becomes re-localized to the plasma membrane upon PT arrival, in a FER-dependent manner, connecting FER to NTA in the same signaling network (Kessler e*t al.* 2010). NTA contains a calmodulin-binding domain in its cytoplasmic C-terminus, possibly allowing synergid cell perception of Ca^{2+} oscillations during PT reception (Iwano *et al.* 2012). Both *lre*/*lre* and *nta*/nta have a similar phenotype to *fer* but not fully penetrant like in the later, suggesting that they are important for PT-synergid interaction but are probably acting redundantly with other unknown factor. *abstinence by mutual consent*, *amc*, also has a phenotype similar to *fer*, but this mutant is self-sterile, that is, the phenotype is observed only when both the PT and the FG carry the *amc* allele (Boisson-Dernier *et al*. 2008). *AMC* encodes a peroxine involved in protein import in peroxisomes, which could be important for the production of small signaling molecule such as ROS or NO, which might be involved in PT-synergid signaling. *TURAN* (*TUN*), encoding an UDP-glycosyltransferase protein was also identified as a possible molecule involved in PT reception, since its mutant as a *fer*-like PT overgrowth phenotype (Lindner *et al.* 2012b). VERDANDI (VDD) is a member of the plant-specific B3 superfamily of transcription factors and *vdd* shows defects in antipodal and synergid cell identity resulting in the absence of PT burst, so growth continues after targeting the synergid cells.

Differently from the mutants described above, the overgrowth phenotype was not observed in *vdd*. VDD may act downstream of these cell surface signaling components (Matias-Hernandez *et al.* 2010).

Not so much is known about the male signalling components involved in PT-synergid interactions. So far, ANXUR1 and 2 (ANX1 and ANX2) were the main components identified as pollen intervenients, being closely related homologues of FER (Boisson-Dernier *et al.* 2009). The two RLKs are localized on the PT tip plasma membrane and redundantly control the timing of PT discharge. Their overexpression inhibits growth by over-activating exocytosis and over-accumulation of secreted cell wall material (Boisson-Dernier *et al.* 2013). Given this, the authors suggested that ANX inhibits PT rupture and sperm discharge at the tip of growing PT constitutively, allowing PT growth within maternal tissues, maintaining their cell wall integrity, until they reach the FG. Once at the FG, the female FER-dependent signaling cascade is activated to mediate PT reception and fertilization, while male ANX-dependent signaling is deactivated, enabling the PT to rupture and deliver its sperm cells (Boisson-Dernier *et al.* 2009; Miyazaki *et al.* 2009). Three pollen expressed transcription factors MYB97, MYB101 and MYB120, were reported to control the expression of genes whose encoding proteins play essential roles in PT-synergid interactions (Liang *et al.* 2013). The single mutants did not reveal any phenotype, only the triple *myb97 myb101 myb120* PTs grow normally along the pistil tissues but revealed uncontrolled growth and failed to discharge sperm cells after entering the synergids. These transcription factors are critical for the PT to exchange signals with the synergids (Liang *et al.* 2013).

In Arabidopsis, receptive synergid degeneration occurs after PT arrival, but before PT discharge (Sandaklie-Nikolova *et al.* 2007) suggesting a PT derived signal to initiate synergid cell degeneration. About the persisting synergid PCD little information is available. According to Schneitz *et al.* (1995), the persistent synergid disappears during the second and third endosperm nuclear division. The molecular mechanisms involved in activation of synergid cell death remains elusive.

Iwano *et al.* (2012) pointed out a possible role for the secondary messenger Ca^{2+} in regulating sperm cell delivery and fertilization. Cytosolic [Ca^{2+}] was shown to be essential for the control of PCD in both PT and the receptive synergid, and for sperm cell fusion with female gametes. Two different studies (Ngo *et al.* 2014; Denninger *et al.* 2014) showed that the PT contact with the receptive synergid, mediated by FER and LRE, initiates oscillations in synergid cytosolic [Ca^{2+}] signatures. These oscillations culminate in a change from [Ca^{2+}] oscillations to a sustained global [Ca^{2+}] flood in the receptive

synergid, until the moment the PT bursts leading to PCD of the receptive synergid, and PT own PCD. NTA is proposed to modulate the intensity of the synergid [Ca^{2+}] signatures in the synergids through its calmodulin-binding site (Ngo *et al.* 2014). Ngo *et al.* (2014) proposes that the persistent synergid either retains its [Ca^{2+}] signature, being responsible for repelling additional PTs after successful double fertilization or reprograms its [Ca^{2+}] signature to that of the receptive synergid upon the arrival of new PTs for rescuing a failed fertilization event. Nevertheless, the signalling events responsible for PCD in the synergid cell, are not understood yet.

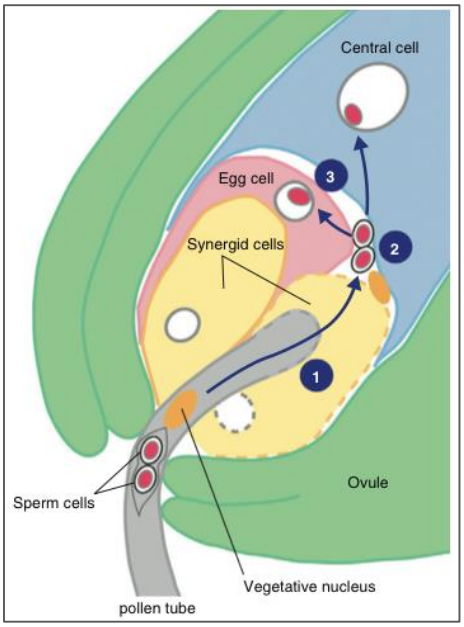

Figure 5 – Schematic representation of pollen tube entrance into the *A. thaliana* embryo sac, sperm cell release into the receptive synergid and its fusion with the female gametes. This process may be divided in three phases:

1. After PT entrance into the receptive synergid the sperm cells are released, and most probably around this time synergid starts degenerating.
2. Sperm cells remain at the boundary region between the egg cell and the central cell for 7.4 minutes and after are directed towards the female gametes.
3. Sperm cells nuclei separate, one is directed towards the central cell nucleus and the other one targets the egg cell nucleus, following karyogamy. Thus, initiating endosperm and embryo development, respectively.

(Image adapted from Hamamura *et al.* 2012).

Following PT entrance into the embryo sac through the receptive synergid there is a rapid transport of the sperm cells by PT discharge to the chalazal pole of the receptive synergid (Fig. 5). Sperm cells adhere to the surface of the female gametes with no preferential order regarding their position in the male germ unit. After this, sperm cells remain immobile for a fairly long period, approximately 7.4 min, in the boundary between the egg and the central cell (Hamamura *et al.* 2011). Afterwards, the membranes of both gametophytes fused (plasmogamy) almost simultaneously, and the two sperm cell nuclei resumed their movement inside each female gamete towards their nuclei (Hamamura *et al.* 2011). During sperm cell fusion with the female gametes three central players have been identified so far: GAMETE EXPRESSED 2 (GEX2) (Mori *et al.* 2014); GENERATIVE CELL SPECIFIC 1/HAPLESS 2 (GCS1/HAP2) (Mori *et al.* 2005; von Besser *et al.* 2006) and the EGG CELL 1 (EC1) family (Sprunck *et al.* 2012). *GEX2* encodes a sperm expressed protein localized to its membrane, containing extracellular immunoglobulin-like

domains, and is required for gamete fusion. In *gex2* sperm cells there is a reduced adhesion to female gametes, likely causing cell fusion failure (Mori *et al.* 2014). *GCS1/HAP2* is predicted to encode a protein with an N-terminal secretion signal, a single transmembrane domain and a C-terminal histidine-rich domain expressed only in the haploid sperm. *gcs1/hap2* sperm cells delivered to ovules fail to initiate fertilization, the released sperm cells remain at the fusion site with female gametes, and end up leading to the attraction of multiple PTs (Mori *et al.* 2005; von Besser *et al.* 2006). EC1, small cysteine-rich protein, is predicted to activate sperm cell for gamete fusion (Sprunck et *al.* 2012). EC1 is stored in vesicles in the egg cell, and secreted to the apical region of the degenerative synergid cell conducting to re-localization of the fusogen GCS1/HAP2 to the cell surface of the sperm cell. Before fertilization GCS1/HAP2 is localized in the endomembrane system (Mori *et al.* 2005). After this, sperm cells are activated and ready to fuse with the female gametes, leading to double fertilization.

In order to prevent multiple fertilization events (polyspermy), that could lead to embryo lethality or malformation of the endosperm, and thus reproductive failure, plants evolved mechanisms to protect them from this risk.

Maruyama *et al.* (2013) showed that the successful fertilization of the egg and the central cell initiates a blocking mechanism mediated by the FIS-PRC2 (Fertilization Independent Seed - Polycomb Repressive Complex 2) complex, to avoid the attraction of multiple PTs (polytubey). By the same time it was also shown the involvement of ethylene in the triggering of the persistent synergid cell death in order to avoid polytubey, involving the transcription factors EIN2 and EIN3 (ethylene-insensitive3-like2). With the death of the persisting synergid, production of PT attractants stopd, leading to the polytubey block (Völz *et al.* 2013). The polytubey blocking system is described in more detail in Chapter 4.

In conclusion, this last step of PT growth including PT burst, sperm cell discharge and fusion with the female gametes along with the polytubey blockage system involves a complex and intricate system of communication and signaling mechanisms between the PT and the female sporophytic and gametophytic tissues, ensuring double fertilization and, therefore, reproductive success.

Arabinogalactan proteins – an everlasting mystery

AGPs are a large family of proteins that are themselves members of a much larger superfamily of proteins, the hydroxyproline-rich glycoproteins (HRGPs). AGPs are the

most glycosylated members of the superfamily, and are usually divided into 4 main sub-families according to their polypeptide core: the classical AGPs, the lysine-rich AGPs (Lys-rich AGPs), the arabinogalactan peptides (AG peptides) and the fasciclin-like AGPs (FLAs) (Seifert and Roberts 2007; Showalter *et al.* 2010). Classical AGPs are characterized by the presence of an N-terminal signal peptide that is removed from the core polypeptide and targets it for secretion, a proline/hydroxyproline-rich core domain, and a C-terminal GPI anchor-addition signal sequence, which is also cleaved to produce the mature protein. The other AGP sub-families are structurally similar to the classical AGPs but either have small mature polypeptides with only 10 to 13 amino acids (AG peptides), or have lysine-rich (Lys-rich AGPs) or fasciclin-like (FLAs) domains in their polypeptide core (Showalter 2001; Schultz *et al.* 2002; Johnson *et al.* 2003). AGPs, like other GPI-anchored proteins, become tethered to the outer layer of the plasma membrane facing the extracellular environment but a few members may not be GPI-anchored (Showalter *et al.* 2010). Two excellent reviews about the structure and general roles of AGPs in plants have been produced, and are highly commended (Gaspar *et al.* 2001; Seifert and Roberts, 2007). The putative mode(s) of action of AGPs have been mostly inferred from their characteristic features, namely high sugar content and association with the plasma membrane through a GPI anchor. One model proposes that the sugars may be cleaved by specific enzymes and released into the extracellular medium where they may act as signalling molecules, morphogens or nutrients (Showalter 2001; van Hengel *et al.* 2001). A second model suggests that the whole AGP might be released by cleavage of the GPI anchor, acting itself in signal transduction pathways, in ways yet to be understood (Schultz *et al.* 1998). One recent study by Lamport and Várnai (2012) suggests that AGPs may act as Ca^{2+} chelators, which would allow the cells to control the release of this ion in specific conditions. These models are not mutually exclusive, and given the complex properties of these macromolecules, it is likely that they may act in diverse modes. Before the development of powerful molecular biology tools that we know today, and the consequent explosion of nucleotide sequence and expression data, much of what we knew about AGPs in reproductive cells resulted either from studies performed with monoclonal antibodies (mAbs) or with the Yariv reagent, which binds specifically to AGPs (Yariv *et al*. 1967). The studies performed with the Yariv reagent essentially provided a functional approach to the study of AGPs, allegedly neutralizing their function, while also being used both as a histochemical and a purification reagent (Seifert and Roberts, 2007). Anti-AGP mAbs have the drawback of being directed mostly to carbohydrate epitopes that are common to various AGPs (Nothnagel, 1997). In any case, and mostly as a result of

the use of these two types of reagents, AGPs are known to have a remarkable distinctive spatial and temporal presence in cell walls, plasma membranes and extracellular secretions of several cell, tissue and organ types (Majewska-Sawka and Nothnagel, 2000).

AGPs in the female reproductive organs

In flowering plants, the immotile sperm cells require a means of transportation to reach the embryo sac, which is nested inside the ovule (Fig. 6). To accomplish this, the PT develops as a protrusion from the PG. Once the PG adheres to the stigmatic cells and hydration starts (Dresselhaus and Franklin-Tong, 2013), the PT begins its growth through the several pistil tissues until it reaches its final destination: the embryo sac.

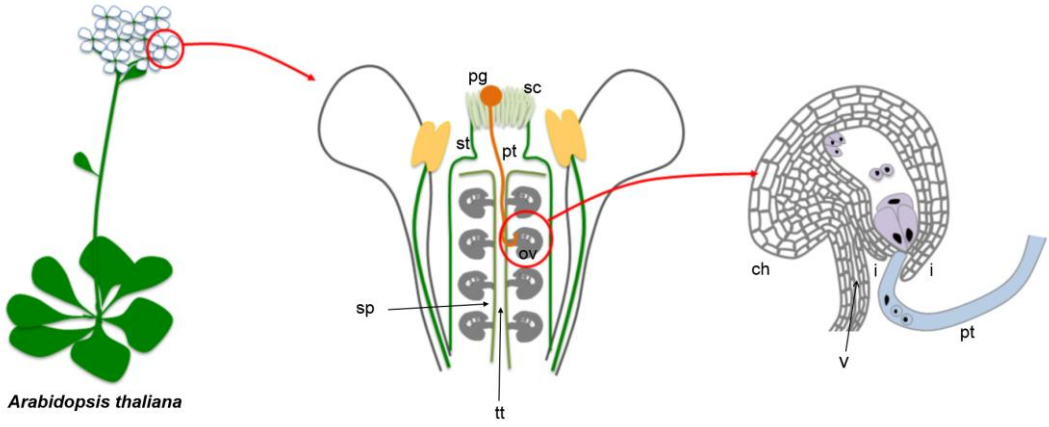

Figure 6 - A schematic representation of the reproductive structures and tissues of *A. thaliana*. Abreviations: ch, chalaza; i, integuments; pg, pollen grain; pt, pollen tube; sc, stigmatic cell; sp, septum; st, style; tt, transmitting tract; v, vasculature. (Adapted from Pereira *et al.* 2014.)

Here, double fertilization takes place: one sperm cell fuses with the egg cell, originating the embryo, while the other fuses with the central cell, initiating endosperm development (Berger *et al.* 2008). All in all, a new seed starts to develop, and a new plant generation is set.

In order for this process to be successful, it is crucial that since the moment the PG contacts the stigma until the double fertilization event, there is a precise and well controlled communication and coordinated growth of the developing PT with the female

sporophytic and gametophytic tissues (Palanivelu and Tsukamoto, 2012). At the outset, the PG must contact with the stigmatic cells and be recognized as compatible to start germinating (Lord, 2002). As the sperm cell carrier, the PT must grow through the TT (Ray *et al*. 1997), a specialized tissue thought to facilitate and nourish the PT development (Faure *et al.* 2002; Crawford and Yanofsky, 2008). The PT grows along this so-called super highway (Johnson and Preuss, 2002) until it makes a quick turn into the septum surface, and grows along the funiculus towards the micropyle, through which the PT reaches the synergids filiform apparatus (SFA) and penetrates through the cell wall of the receptive synergid, bursting it and itself, and releasing the two sperm cells to accomplish the double fertilization (Johnson and Preuss 2002; Dresselhaus and Franklin-Tong 2013). This whole process is described in more detail in the sections above. AGPs have been detected throughout all these tissues in a temporal and spatial differential pattern.

AGPs in megasporogenesis and megagametogenesis

The Arabidopsis embryo sac follows the *Polygonum* type of development (Schneitz *et al.* 1995). Initially, in the nucellus of the ovule primordium, an archesporial cell differentiates into a MMC, which undergoes meiosis and originates four megaspore cells. Only one of them survives, becoming the functional megaspore (FM). This FM will, in turn, give rise to the FG after a series of syncytial mitotical divisions and cellularization, which culminates with the formation of a seven-celled eight-nucleate mature embryo sac, with three antipodal cells localized in the chalazal pole, a double-nucleated central cell, and one egg cell surrounded by two synergids (Drews and Yadegari, 2002; Ma and Sundaresan, 2010). Pennell and Roberts (1990) proposed that the presence or absence of arabinose-containing epitopes recognized by the mAb MAC207 (Pennell *et al.* 1989) would be a “presage” for the switch from a vegetative to a reproductive stage of development in *Pisum sativum*. These AGP epitopes were described only in the sporophytic tissues preceding the establishment of the gametophytic lineage. Later, Pennell *et al.* (1991) characterized a new mAb, JIM8, which recognized AGP sugar epitopes present also during the gametophytic phase of development, namely in the sperm and egg cells and in the SFA in *Brassica napus* flowers. Coimbra *et al.* (2007) using several anti-AGP mAbs, were able to detect AGP-specific sugar epitopes in the FM (Fig. 7A) as well as in the FG, specifically in the synergid cells and SFA of Arabidopsis (Fig. 7B, C), validating the immunolocalization studies performed by Pennell and Roberts

(1990) and Pennell *et al.* (1991) and the concept of differential expression of AGPs in plant reproductive tissues.

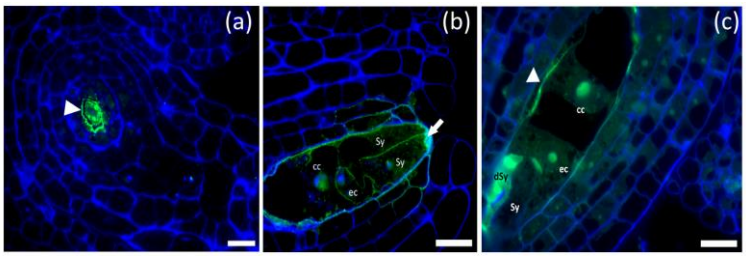

Figure 7 - Fluorescence microscopy of Arabidopsis pistil tissues at different stages of ovule development, labelled with mAbs specific for AGPs (JIM8 and JIM13) with FITC-conjugated secondary antibody. (a) Final stage of megasporogenesis, with the surviving functional megaspore visible. The epitopes recognized by JIM13 are specifically labelling the entire functional megaspore surface (arrowhead). (b) Labelling with JIM8 of all gametophytic cell walls and the filiform apparatus (arrow). (c) The labelling with JIM8 is present in the embryo sac wall (arrowhead), delimits the synergid cells and is present in the central cell and egg cell. Abreviations: cc, central cell; ec, egg cell; dSy, degenerated synergid; Sy, remaining synergid. Scale bars = 5 µm.

In an Arabidopsis RNAi line for the Lys-rich *AGP18* gene, the FG fails to enlarge and divide mitotically (Acosta-García and Vielle-Calzada, 2004), suggesting that AGP18 may be essential for a proper gametogenesis in Arabidopsis. More recently, Demesa-Arévalo and Vielle-Calzada (2013) determined the pattern of AGP18 localization in ovules by introducing an epitope at the N-terminal end of the AGP18 mature polypeptide, and analysed the respective transgenic plants by immunolocalization. The authors did not detect AGP18 in the four initial haploid megaspore cells resulting from the MMC meiotic division, but detected it abundantly in the nucellar and integumentary cells, which was consistent with the prior immunolocalization results. However, they found AGP18 to be present in the FM and in the FG, more precisely in the central cell and the egg apparatus. Because the presence of AGP18 transcripts could be detected in the MMC by *in situ* hybridization (Acosta-García and Vielle-Calzada, 2004), the authors concluded that AGP18 mRNA was not able to be transcribed in the MMC, unless meiosis occurred. It was also suggested, based on immunolocalization studies with JIM13, that the post-translational glycosylations of AGP18 occurred only in the FM, where the antibody recognized the sugar epitopes. The referred study supports the hypothesis that each cell or tissue type, somatic or reproductive, may have different glycosylation machineries that determine cell fate, as indicated before by Pennel *et al.* (1991). The experiments with AGP18 are a remarkable example of how new molecular tools complemented earlier studies performed with mAbs.

Tucker *et al.* (2012), and Tucker and Koltunow (2014) also highlighted the importance of other AGPs, such as AGP22 and AGP24, in MMC and FM formation and development. Yellow fluorescent protein expression driven by the AGP22 and AGP24 promoters was directed to the nucellar epidermal cells and to the FM, respectively, supporting the immunolocalization studies made with JIM13 by Coimbra *et al.* (2007). Functional studies with AGPs have been scarce despite new molecular and imaging tools, which is bound to be a consequence of the complexity of AGPs.

AGPs and PT growth through the stigma, style and transmitting tract

With grains and fruits comprising over 90% of the world agricultural production (FAO, 2014), it can safely be said that double fertilization in flowering plants is a paramount subject for humankind. In the last two decades significant advances have been achieved regarding the understanding of the mechanisms involved in sexual plant reproduction. Several recent reviews give us an excellent update about all these new findings (Beale and Johnson, 2013; Dresselhaus and Franklin-Tong, 2013; Bleckmann *et al.* 2014). It has been more than thirty years since AGPs were first identified and implicated in Angiosperm reproduction. The first studies were done with the aid of β-glucosyl Yariv reagents or with anti-AGP mAbs. The presence of AGPs along the pathway followed by the PT inside the pistil tissues until it reaches the embryo sac have been documented in numerous and phylogenetically diverse plant species [Gleeson and Clarke, 1980; Hoggart and Clarke, 1984; Sedgley *et al*. 1985; Webb and Williams, 1988; Jauh and Lord, 1996; Coimbra and Salema, 1997; Lennon *et al.* 1998; Cheung *et al.* 2000; Wu *et al.* 2000; Coimbra and Duarte, 2003; Peng *et al*. 2005; Coimbra *et al.* 2007; Losada and Herrero, 2012, 2014; Costa *et al.* 2013b; Suárez *et al.* 2013 (for a more detailed description see Table 1)].

Table 1 – Distribution of AGPs in female reproductive tissues in a variety of plant species

Species	Reproductive tissues	Detection by:		Molecular Biology Techniques	References
		Immunolocalization	Yariv reagent		
A. thaliana	TT, inner integument, embryo sac, FM, SY, filiform apparatus	JIM8, JIM13, MAC207	-	-	Coimbra *et al.* 2007
A. thaliana	TT ECM	JIM8, JIM13, MAC207	Histochemistry	-	Lennon *et al.* 1998

Species	Reproductive tissues	Detection by:		Molecular Biology Techniques	References
		Immunolocalization	Yariv reagent		
A. thaliana	Nucellar and integumentary cells, FM, central cell, egg apparatus, AGP18 transcripts in the MMC	JIM13	-	***AtAGP18*** RNAi knock-down, *in situ* hybridization, promoter fusion analyses (GUS)	Acosta-García & Vielle Calzada 2004 Demesa-Arévalo & Vielle-Calzada 2013
A. thaliana	nucellar epidermal cells - ***At*AGP22** FM – ***At*AGP24**	JIM13	-	***At*AGP22** & ***At*AGP24** Promoter fusion analyses (YFP)	Tucker *et al.* 2012 Tucker & Kultonow 2014
A. thaliana	Style, ovary walls, TT, siliques	-	-	***AtAGP19*** knock-out mutant, Northern blot, promoter analyses (GUS)	Yang *et al.* 2007
A. thaliana *A. thaliana*	Stigma, TT, funiculus, integuments, chalaza – ***At*AGP1** Ovary vasculature – ***At*AGP9** Stigma, septum, funiculus, chalaza – ***At*AGP12** All pistil tissues except TT – ***At*AGP15**	-	-	***At*AGP1, *At*AGP9, *At*AGP12, *At*AGP15** Promoter analyses (GUS & GFP), *in situ* hybridization, microarray data analyses, Real Time RT-PCR	Pereira *et al.* 2014
P. sativum	Nucellar and integumentary cells	MAC207	-	-	Pennell & Roberts 1990
B. napus	Nucellar epidermis, egg cell, SY, filiform apparatus	JIM8	-	-	Pennell *et al.* 1991
L. longiflorum	TT ECM	JIM13, MAC207	Precipitation, immunoblot, Yariv treatment	-	Hoggart & Clarke 1984; Jauh & Lord 1996
G. gandavensis	Stigma surface, style mucilage	-	Precipitation, affinity chromatography	-	Clarke *et al.* 1979; Gleeson & Clarke 1980; Hoggart & Clarke 1984
L. peruvianum	Ovary & style mucilage, mucilage of the placental surface & between	-	Sections stainning		Webb & Williams 1988

Species	Reproductive tissues	Detection by:		Molecular Biology Techniques	References
		Immunolocalization	Yariv reagent		
	ovules				
L. esculentum	TT ECM	Anti-*Le*AGP1	Precipitation	***Le*AGP1** gDNA, cDNA & protein characterization, mRNA expression pattern, *Le*AGP1 overexpression	Gao *et al.* 1999; Gao & Showalter 2000; Sun *et al.* 2004
N. alata	Stigma, style, TT ECM	mAbs PCBC3, PCBC1	Quantification by radial diffusion, precipitation	-	Gell *et al.* 1986, 1995; Hoggart & Clarke 1984; Sedgley *et al.* 1985
N. alata	Style, stigma of mature flowers	-	Gel diffusion assay	**AGP*Na*1** & **AGP*Na*3** Northern blot, *in situ* hybridization, cDNA & protein analyses	Du *et al*. 1994; Du *et al*. 1996
N. alata *N. alata*	TT, basipetal increase in glycosylation - ***Na*TTs** TT, evenly distributed in the ECM, incorporated by PTs – **120 kDa**	Anti – *Ga*RSGP, JIM13, anti – 120 kDa	Immunoblot	***Na*TTS** & **120 kDa** Northern blot, *in situ* hybridization, sugar composition analyses, cDNA & protein analyses	Chen *et al.* 1993; Sommer-Knudsen *et al*. 1996; Wu *et al.* 2000; Lind *et al.* 1994, 1996; Schultz *et al.* 1997
N. tabacum	TT with basipetal increase in glycosylation, incorporated by growing PTs	Anti-PELPIII	Yariv treatment, immunoblot	**TTS** & **PELPIII** cDNA library screening, protein & sugar composition analyses, *knock-down* mutant	Cheung *et al*. 1995; Wu *et al*. 1995; Goldman *et al.* 1992; Bosch *et al.* 2001; Eberle *et al.* 2013
N. tabacum	Egg cell	-	Yariv treatment	-	Qin & Zhao 1996
A. hypocondriacus	stigma, TT, mycropilar nucellus, embryo sac, SY, filiform apparatus	MAC207, JIM8, JIM13	-	-	Coimbra & Salema 1997; Coimbra & Duarte 2003
A. deliciosa	Stigma, TT exudate, obturators, embryo sac	MAC207, JIM8, JIM13	-	-	Coimbra & Duarte 2003

Species	Reproductive tissues	Detection by:		Molecular Biology Techniques	References
		Immunolocalization	Yariv reagent		
	cells and wall				
T. submersa	Ovary wall, outer integument, stigmatic hairs, nucellus tissue at the entrance of the embryo sac	MAC207, JIM8, JIM13	-	-	Costa *et al.* 2013b
C. sativus	Carpel & pistil primordium	MAC207, JIM8	-	-	Peng *et al.* 2005
O. europea	stigmatic exudate, stigma secretory cells, TT	JIM13	-	-	Suárez *et al.* 2013
Malus x domestica	Stigma secretions	JIM8, JIM13	-	-	Losada & Herrero 2012, 2014
P. hybrida	Stigma, style	-	Yariv stainning	***Ph*PRP1**, ortolog of *Nt*TTS, protein immnoblot analysis, SDS-PAGE	Twomey *et al.* 2013
L. decidua Mill	Nucellus, mature archegonium, egg cell cytoplasm	JIM8, JIM13	-	-	Rafińska & Bednarska 2011
G. nivalis, G. candicans, Oenothera & Sinapsis alba	Egg apparatus, somatic mycropilar tissues	JIM8, JIM13	-	-	Chudzik *et al.* 2005

(Abreviations: ECM, extracellular matrix; FM, functional megaspore; MMC, megaspore mother cell; SY, synergids; TT, transmitting tissue)

Recently, Kitazawa *et al.* (2013) found that the Yariv reagent binds in a specific way to the β-1,3-galactoligosaccharides of AGPs, which may help us understand the mode of action of AGP oligosaccharides, and whether or not they may be acting as signalling molecules, as proposed by some authors (Showalter, 2001; van Hengel *et al.* 2001). It remains to be determined whether a specific sugar component of the AGP is the part directly involved in a proposed developmental process, or if the binding of the Yariv reagent to the sugar chains is only modifying the AGP overall structure, hindering these molecules from undertaking their respective functions.

The use of the Yariv reagent and mAbs, although still very useful for AGP studies, do have some weaknesses, like the non-recognition of a specific AGP. It is essential to identify a specific AGP in order to study its structure, gene expression and correlate it to function in more detail. Gao *et al*. (1999) and Gao and Showalter (2000) conducted a detailed study to immunolocalize *Le*AGP1 in tomato plants by using a specific antiserum raised against the *Le*AGP1 15-amino acid lysine-rich domain, which being proline-free is also sugar-free. Indeed, the probability of successfully generate specific antibodies for other AGPs is very low due to their high degree of glycosylation (Schultz *et al.* 2000). *Le*AGP1 was shown to be present in the ECM of the stylar TT, and shown to have higher levels of glycosylation in immature flowers compared to mature flowers, suggesting that these changes could be related to the pollen germination and PT growth through the TT, similarly to what has been found for Nicotiana species (Cheung *et al.* 1995), and which are described in more detail later. Sun *et al.* (2004) studied transgenic tomato plants overexpressing *Le*AGP1 variants fused with green fluorescent protein (GFP), and two other plant lines, one lacking the C-terminal GPI-anchor domain (GFP-*Le*AGP-1ΔC) and the other lacking the lysine-rich domain (GFP-*Le*AGP-1ΔK), observing the production of smaller but viable seeds and reduced fruit production in plants overexpressing *Le*AGP1 and in GFP-*Le*AGP-1ΔK plants. In contrast, the effect of *Le*AGP-1ΔC overexpression on plant reproduction was minimal, which suggests that the GPI anchor is central to *Le*AGP1 function. More recently, Fragkostefanakis *et al.* (2012) searched the tomato genome database for AGPs related to fruit ripening and found 34 genes encoding putative AGPs, from which at least 20 of them were expressed in fruits. The expression profiles of 3 specific AGPs, *Le*AGP1, *Sl*AGP2 and *Sl*AGP4 were analysed during fruit ripening, and in response to hypoxia, anoxia and mechanical wounding. *Sl*AGP2 was up-regulated during fruit ripening following the climacteric ethylene production. *Sl*AGP4 and *Le*AGP1 mRNA levels were higher in response to mechanical wounding, while under anoxia only SlAGP4 was up-regulated. Tomato AGPs appear therefore to play important roles in fruit ripening and in response to different stress conditions.

Despite the fact that anti-AGP antibodies do not recognize individual molecular species, they may still be extremely useful in plant species for which there is still little or no sequence information available. For example, in *Olea europaea* pistil tissues, Suárez *et al.* (2013) detected the presence of AGPs by using mAb JIM13, which revealed the presence of higher amounts of AGPs during pollination when compared to pistils at the pre-anthesis stage and after pollination. This study supports the view by which AGPs are important to the growth of PTs along the pistil tissues. Similar studies were carried out by

Losada and Herrero (2012) in *Malus x domestica*. Using mAbs JIM8 and JIM13 the authors concluded that the acquisition of stigma receptivity in the apple flower was dependent on secretion of AGPs, and that the disappearance of these AGPs is the stigma after pollination was related to the PT growth. Furthermore, AGPs were not found to be present in the stylar TT, and this change in the presence of AGP epitopes was correlated with a slower growth of the PT in the stigmatic tissues and a faster growth of the PT in the stylar tissues, suggesting that differences in the sugar epitopes detected along the PT pathway could explain the different rates of PT growth (Losada and Herrero, 2014).

AGPs in Nicotiana tabacum and N. alata pistil tissues – cutting a long story short

Some of the better studied AGPs at the molecular level are from Nicotiana species. Transmitting Tissue Specific (TTS) AGPs from tobacco were the first AGPs to be described in detail, and were shown to stimulate PT growth in vitro and to attract PTs in a semi in vivo assay (Cheung *et al.* 1995; Wu *et al.* 1995). TTS proteins were suggested not only to support PT growth along the stigma and ovary but also to guide the PTs along its journey to the embryo sac. Furthermore, transgenic plants with reduced levels of TTS proteins correlated with a reduced PT growth rate (Cheung *et al.* 1995). An important feature revealed by these studies was the basipetal increase in glycosylation of these proteins in the pistil, which might be related to PT guidance. PTs deglycosylate TTS proteins, suggesting that these may act as a nutrient factor positively affecting PT growth (Cheung *et al*. 1995; Wu *et al.* 1995). Twomey *et al.* (2013) identified an ortholog of a tobacco TTS protein gene in *Petunia hybrida* pistils (*PhPRP1*) that is not only present in the TT, but is also expressed in vegetative tissues, and thus not pistil tissue-specific. *Ph*PRP1 also does not show the same basipetal gradient of increasing glycosylation, and has no effect on PT growth in vitro or in semi in vivo experiments. The precise role of this protein remains unclear.

In *N. alata* the *Na*PRP4 (Chen *et al.* 1993) and the GaRSGP – Galactose Rich Stylar GlycoProtein – (Sommer-Knudsen *et al.* 1996) have been demonstrated to be equivalent to the *Na*TTS proteins identified by Wu *et al.* (1995). These proteins are similarly localized in the style TT ECM, they also stimulate PT growth in vitro and attract PTs in semi in vivo assays, and show a basipetal increase in glycosylation levels in *N. alata* pistils. A chimeric 120 kDa AGP purified from *N. alata* styles, and shown to contain

both extensin-like and AGP-like side chains, was found to be style-specific and uniformly distributed along the ECM of the TT (Lind *et al.* 1994; Schultz *et al.* 1997). One important characteristic of this glycoprotein was its incorporation into the cytoplasm and cell wall of PTs during in vivo growth through the pistil tissues, but also in vitro when the 120 kDa glycoprotein was introduced into the growth medium (Lind *et al.* 1996). Hancock *et al.* (2005) suggested the involvement of this protein with S-specific pollen rejection phenomena, in self-incompatibility mechanisms. The 120 kDa glycoprotein was shown to interact with the S-RNase-binding protein (SBP1), a C2 domain-containing protein (*Na*PCCP), and a putative cysteine protease (Lee *et al.* 2008), in *N. alata*. The authors suggested that the binding between *Na*PCCP, *Na*SBP1 and the pistil AGPs may contribute to signalling and trafficking inside PTs growing in planta (Lee *et al.* 2009).

In a study of identification of genes specifically expressed in the pistil of tobacco, Goldman *et al*. (1992) found that the genes *MG14* and *MG15* encoded a class of Pistil-specific Extensin-Like Proteins (PELPIII), which localized specifically to the pistil TT, and showed a basipetal increase in glycosylation until the time of pollination, after which its levels started to decrease. Bosch *et al.* (2001) showed that these proteins actually possess AGP-like glycan moieties, being considered chimeric AGPs, similarly to the 120 kDa glycoprotein of *N. alata*. PELPIII's were shown to be translocated from the ECM of the pistil to the callosic layer and callose plugs of the PTs in vivo, but unlike the 120 kDa glycoprotein, it was not translocated into their cytoplasm (de Graaf *et al.* 2003). Recently, in a very elegant study, Eberle *et al.* (2013) demonstrated the importance of PELPIII's in pre-zygotic interspecific incompatibility. In this study, by pollinating TT-ablated tobacco pistils with pollen from *N. obtusifolia* and *N. repanda*, the interspecific PTs were able to grow, thus losing the interspecific incompatibility. Therefore, the authors hypothesized that some protein produced and secreted by the TT into the ECM should be responsible for the inhibition of interspecific PT growth. Following this assumption, they injected tobacco TT extracts into the TT-ablated pistils, restoring their ability to inhibit the growth of interspecific PTs. Furthermore, pistils with anti-sense suppression of PELPIII's confirmed these results and the importance of PELPIII accumulation in TT of tobacco to avoid interspecific PT growth of *N. obtusifolia* and *N. repanda*.

Taking into account the abundance of these proteins along the stigma and style of these species, as well as the incorporation of some of these glycoproteins into the growing PTs it is reasonable to assume that AGPs are involved in pollen recognition, adhesion, PT nutrition and guidance along the pistil tissues.

AGPs in Arabidopsis pistil tissues – wishing it will become a long story

In Arabidopsis, the plant genetic model system par excellence, it could be expected much more information to be available about AGP functions, but indeed it is hardly the case. Schultz *et al. (*2002) published an excellent study on the use of new genomic resources to analyse and better understand AGPs in Arabidopsis. Although it presents several ways to study AGPs at the protein, transcriptomics, and sugar composition levels, it also shows the difficulties inherent to the study of a big family of proteins such as the AGP family. The study of T-DNA insertion mutants is rarely effective, given the possibility of gene redundancy, and to abolish a specific function it is often needed to obtain double, triple or even quadruple mutants. This is not likely to be advantageous and it is extremely time-consuming. Schultz *et al.* (2002) also underlined the importance of testing AGP gene responses to different biotic and abiotic conditions in order to understand AGP functions. The scenario become even more complicated when the study of closely related AGPs reveals that they might have completely different functions (Pereira *et al.* 2014).

AGP19, a Lys-rich AGP, was characterized in terms of genetic expression and function by Yang *et al.* (2007). Promoter analysis using the GUS (β-glucuronidase) reporter gene showed that *AGP19* is expressed in the style, ovary walls, TT and siliques. No GUS activity could be detected in anthers, so it seems to be restricted to female tissues. The study of knock-out mutants for *AGP19* showed a reduction in flower production as well as fewer and shorter siliques than in the wild-type plants.

One of the most recent studies on AGPs in reproductive tissues describes the differential expression pattern of AGP1, AGP9, AGP12 and AGP15 along the Arabidopsis female reproductive tissues using promoter analysis with GUS and GFP, *in situ* hybridization, microarray data analysis and Real Time (RT)-PCR (Pereira *et al.* 2014; see Chapter 3). *AGP1* showed expression in the stigmatic cells, style and in the TT. *AGP9* was found along the vasculature of the reproductive tissues and *AGP12* was expressed in the stigmatic cells and in the septum. *AGP15* was expressed in all pistil tissues, except in the TT. The presence of these AGPs in these tissues resembles the same pattern of expression of AGPs in tobacco pistils. They are present along the pathway of the PT and their functions might be similar to the ones speculated for the Nicotiana AGPs. Further studies are needed in order to identify the specific function of each of these AGPs but it is predictable that they might be acting in recognition and adhesion of PG to the stigmatic cells, and in PT growth sustenance and/or as PT attractants.

AGPs and the final stage of PT growth – ovular and micropylar growth

The final stage of PT growth is currently one of the most studied, but still one of the least understood. The growth of the PT into the micropyle of the embryo sac until it enters a synergid and bursts releasing the sperm cells is designated by micropylar guidance (Shimizu and Okada, 2000). It is still unclear how the PT abruptly changes its growing direction and turns towards an unfertilized ovule. In young flowers of *Lycopersicon peruvianum*, it was observed that AGPs were present in the mucilage of the placental surface and between the ovules (Webb and Williams, 1988). Pennel *et al.* (1991) detected AGPs in the nucellar epidermis, synergid cells and the egg cell of *B. napus*. Coimbra and Salema (1997) and Coimbra and Duarte (2003), studying *Amaranthus hypocondriacus*, identified the presence of AGPs in the cells of the nucellus and integuments surrounding the PT growth pathway to the embryo sac, as well as the presence of AGP epitopes in the egg apparatus (the egg cell and the two synergids). This is exactly the path taken by the PT while it grows between the ovules until it reaches an embryo sac.

Chudzik *et al.* (2005) studied the presence of AGPs in different types of ovules from *Galanthus nivalis*, *Galtonia candicans*, *Oenothera* and *Sinapis alba*. In all the fertile and mature ovules considered, AGPs were present specifically in the egg apparatus and in the distal segment of PT pathway, which the authors correlated with ovule receptivity. Curiously, when analysing ovules without embryo sacs from *G. nivalis* and *O. mut. brevistylis*, no AGPs were detected in the somatic micropylar tissues, suggesting that there must be some communication between the egg apparatus and the nucellar and integumentary tissues surrounding the micropylar entrance to induce the expression of AGPs. The specific presence of AGPs in these tissues during the stage of highest ovule receptivity led the authors to speculate that AGPs might be acting as signalling molecules in this last stage of PT guidance, as it had been already suggested by Coimbra and Salema (1997). Qin and Zhao (1996) also identified higher levels of AGPs in unfertilized egg cells than in fertilized ones in tobacco. The use of Yariv reagent in ovules arrested the fertilization in egg cells but not in the central cells, suggesting that AGPs might be essential only for egg cell – sperm cell recognition. Some studies have also been carried out in ovules of the gymnosperm *Larix decidua* (Rafińska and Bednarska, 2011). AGPs were identified in the nucellus and in the mature archegonium, and in the cytoplasm of the egg cells by immunolocalization techniques. In this species the PT grows directly into the

egg cell; the authors deduced that AGPs could be important for the recognition between the released gamete and the egg cell, and that the presence of AGPs in the nucellus might prepare these tissues to receive the PT, in a similar way to what occurs in the Angiosperm TT. In Arabidopsis, specific AGPs were localized in the funiculus and the ovules (Pereira *et al.* 2014; see Chapter 3). *AGP1* was shown to be expressed in the funiculus, the integument cells near the micropyle and the chalazal tissues of the ovules, *AGP12* was also expressed in the funiculus and the chalazal pole of the ovules, while *AGP15* was expressed in all funiculus, integuments and ovule cells, except inside the embryo sac. Again, the presence of these AGPs in the distal pathway of the PT suggests their importance in some sort of PT support and guidance into the embryo sac to achieve double fertilization.

AGPs in male reproductive organs

In the male counterpart, the reproductive process starts with the development of the anthers and, inside them, the formation of the microgametophyte (the three-celled PG). The anthers comprise the epidermis, the endothecium, the middle layer and the tapetum, all surrounding the pollen mother cells (PMC or microsporocyte). This PMC undergoes meiosis to generate a tetrad of microspores separated from each other by a callose envelope. After microspore release from the tetrads they undergo two rounds of mitosis. The first mitosis is asymmetrical, originating a PG with a vegetative cell that almost engulfs the generative cell. The latter will be the target for the second mitosis, when sperm cells are formed. This second mitosis may take place inside the anther or inside the growing PT, depending on the species, and thus establishing the mature MG or PG, composed by a vegetative cell containing the two sperm cells – the male gametes (Ma and Sundaresan, 2010; see above for a more detailed escription).

Antibodies, such as JIM8, JIM13, JIM14, LM2 and MAC207 were also extremely helpful in providing a developmental map of AGP distribution in Arabidopsis male reproductive cells and tissues (Pereira *et al.* 2006; Coimbra *et al.* 2007; Dardelle *et al.* 2010; Coimbra and Pereira, 2012). These works lent further support to the hypothesis that AGPs are spatially and developmentally regulated, and may be used as precise molecular markers for specific developmental stages of the reproductive development. In Arabidopsis, major hallmarks of the development of the male cell lineage, namely transition from a sporophytic genetic program to a gametophytic genetic program, asymmetric cell division during pollen development, and emergence of the PT, are

observed to show a relationship with similarly major rearrangements of AGP epitopes (Pereira *et al.* 2006; Coimbra *et al.* 2007; Coimbra and Pereira, 2012).

AGPs in non-model species male reproductive organs

Anti-AGP mAbs have been successfully used to study pollen and PTs of many species besides Arabidopsis (reviewed by Nguema-Ona *et al.* 2012), and continue to be so. In olive pollen, for instance, it was found that AGPs were newly synthesized and increased in concentration during pollen germination, and the authors concluded that AGPs, along with pectins, are spatially and developmentally regulated (Castro *et al.* 2013). In pollen of *Trithuria submersa*, an early-divergent Angiosperm, it was observed an intense labelling with anti-AGP mAbs both in the anthers and in the intine wall, the latter associated with PT emergence. AGPs are also speculated to play a significant role in Trithuria reproduction, due to their specific presence in the PT pathway (Costa *et al.* 2013b; see Chapter 2). These results agree with older immunolocalization studies obtained for Arabidopsis (Coimbra *et al.* 2007). In *Quercus suber*, pollen-expressed AGP genes were annotated and correlated with studies of the microgametogenesis with anti-AGP antibodies (Costa *et al.* 2014); in this study, an intense labelling was obtained in the tapetum and in the intine wall near the apertures and, in more advanced developmental stages, also in the generative cell wall and vegetative cell. In *B. napus* the dynamics of AGP epitopes could be correlated to pollen maturation, while another set of AGP epitopes seemed to be associated with the modification of the microspore developmental programme. Still another set of AGP epitopes were associated with microspore derived embryo differentiation, again suggesting that AGPs may be acting as signalling and/or regulatory molecules (El-Tantawy *et al.* 2013).

In rice it has been recently shown that male reproductive cells specifically express and secrete the product of MTR1 gene, a fasciclin glycoprotein (akin to the fasciclin-like AGPs of Arabidopsis), and a mutant of this gene showed male sterility caused by defects not only in the normal development of the microspores but also in adjacent somatic cells (Tan *et al.* 2012). Some of the more recent studies are summarized in Table 2.

Table 2 – Distribution of AGPs in male reproductive tissues in a variety of plant species after 2012*

Species	Reproductive tissues	Detection by:		Molecular Biology Techniques	References
		Immunolocalization	Yariv reagent		
A. thaliana	Vegetative cell of the PG Growing PT	-	-	***At*AGP23** Promoter analyses (GUS & GFP), microarray data analyses, Real Time RT-PCR	Pereira *et al.* 2014
O. europea	Generative cell wall, outer exine layer of PG, germinative aperture, cell wall of the emerging PT, PT cell wall	JIM13, JIM14	-	-	Castro *et al.* 2013
T. submersa	Anthers, intine wall of PG, local of PT emergence	MAC207, JIM8, JIM13	-	-	Costa *et al.* 2013b
Q. suber	Tapetum, intine wall, germinative aperture, generative cell wall, vegetative cell	MAC207, JIM8, JIM13	-	***Qs*AGP15** & ***Qs*AGP23** Transcriptomic analyses, cDNA analyses, Real Time RT-PCR	Costa *et al.* 2014 (in press)
B. napus	Mature PG, cytoplasm & wall of generative cell & sperm cells, vegetative cell	JIM13, JIM14, MAC207	-	***Bn*AGP Sta39-4** Analyses of gene expression pattern of *B. napus* gene encoding AGP	El-Tantawy *et al.* 2013
O. sativa	Male reproductive cells plasma membrane: microsporocytes & tetrads	Anti-MTR1	-	**MTR1** Mutant analyses, expression pattern, Real Time RT-PCR, *in situ* hybridization, phylogenetic analyses	Tan *et al.* 2012

* Detailed information about data published before 2012 reviewed by Nguema-Ona *et al.* 2012.
(Abreviations: PG, pollen grain; PT, pollen tube)

AGPs in Arabidopsis male reproductive organs

The most representative Arabidopsis pollen AGPs are AGP6, AGP11, AGP23, AGP40 and FLA3, all pollen specific, and also the non-pollen specific AGP15, AGP21, AGP22 and AGP24 (Lalanne *et al.* 2004; Pina *et al.* 2005; Pereira *et al.* 2006).

By using the Arabidopsis *AGP6* promoter to drive GFP expression, it was shown that its expression was absent in all vegetative plant tissues but became evident just after the appearance of the locules in anthers (which corresponds to stage 9 of development as described by Smyth *et al.* 1990). GFP fluorescence was limited to pollen and PTs and could be clearly differentiated from the autofluorescence typical of the exine and of the endothecium lignin thickenings. This signal persisted in mature PGs and growing PTs (Coimbra *et al.* 2008, 2009). Gene fusions of AGP6 with SUFP (sirius ultramarine fluorescent protein) showed that the protein is present in the vegetative cell wall only, apparently being excluded from the generative cell wall of the PT (Pereira *et al.* 2013).

Null mutants for single AGP genes (namely *agp6*, *agp11*, *agp40*) have been shown to lack obvious phenotypic differences when compared to wild-type Arabidopsis (Levitin *et al.* 2008; Coimbra *et al.* 2009). However, the double *agp6 agp11* null mutant did produce remarkable phenotypic traits, which included failure of PGs to develop normally, reduction in pollen germination and PT growth rate, and premature pollen germination (Coimbra *et al.* 2009, 2010). The collapsed PGs had degenerated contents, seen by scanning electron microscopy and transmission electron microscopy. All of this indicated that these genes are crucial for pollen and PT development. The “inside the anthers” premature germination phenotype of *agp6 agp11* was air humidity-dependent, and was never observed in wild-type plants nor in any of the two *agp6* or *agp11* single mutants (Coimbra *et al.* 2010). AGP6/AGP11 thus seem to play a role in preventing an early and wasteful germination of pollen inside the anthers, and suggests that AGPs may be interfering with the control of pollen germination, maybe by a relatively simple process of modulating access of water for hydration (or for the earlier dehydration process), or by interfering with some kind of signalling pathway. A triple *agp6 agp11 agp40* mutant showed a more penetrant phenotype than the double *agp6 agp11* mutant and an even lower seed set (Nguema-Ona *et al.* 2012). A similar phenotype of pollen abortion, yet showing partial seed viability, was obtained in knock-down Arabidopsis RNAi plants for the FLA3 gene which, likewise, suggest that FLA3 may be involved in normal microspore development. Furthermore, FLA3-overexpressing transgenic plants resulted in defective elongation of stamen filaments, shorter siliques and reduced seed set, which also suggests that FLA3 may affect the normal plant growth pattern (Li *et al.* 2010).

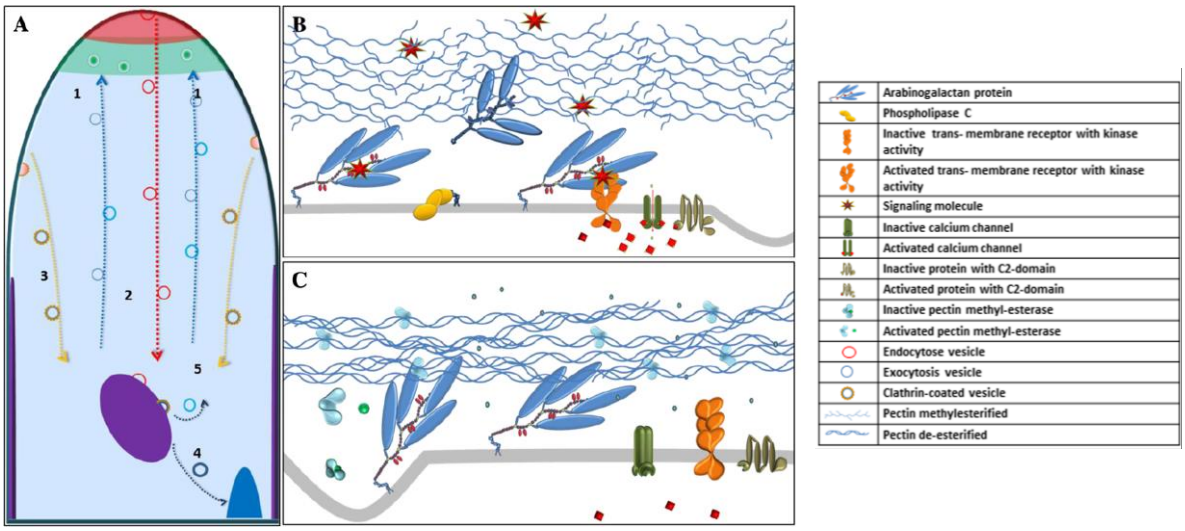

Figure 8 - A model for the role of AGPs in pollen tube growth. (a) Accepted model for pollen tube growth (Zhao et al. 2010; Bove et al. 2008), illustrating vesicle exocytosis (1), smooth endocytosis (2), clathrin vesicle endocytosis (3), the pathway into degradation (4) and the recycling pathway (5). The apical dome (red), the exocytosis region (green), the multivesicular body (purple) and the vacuole (blue) are shown. (b) Proposed model for AGP signaling role in the pollen tube apical dome. AGPs act as receptors for extracellular signals and interact with transmembrane proteins, possibly receptor kinases or C2 domain-containing proteins. These interactions lead to opening of calcium channels, triggering various intracellular events. During pollen tube growth, AGPs are recycled by endocytosis, and either reused or sent for degradation, through multivesicular bodies. (c) Proposed model for the sub-apical zone. The hardening of cell wall pectins resulting from complexing with calcium hinders AGP contact with putative signal molecules, rendering AGPs inactive. (Adapted from Costa *et al*. 2013a)

A microarray data analysis of the *agp6 agp11* double mutant produced a high number of genes with altered expression, in particular the differential expression of signalling-related and Cys-rich protein genes. This reinforced the idea that these molecules are involved in complex phenomena (Costa *et al.* 2013a), since these variations are consistent with the known characteristics of PT growth, and Cys-rich proteins having been associated with recognition and fertilization phenomena. On the same study, an Y2H assay for putative interacting gene partners of AGP6 resulted in a small set of proteins such as a calcium-dependent lipid-binding family protein, MAPK9 kinase, polyubiquitin UBQ10, and the transcription factor FBR12, and for AGP11, AP2C1, a putative protein phosphatase. From both experiments it was hypothesised that AGP6/AGP11 may be involved in signalling, endocytosis and secretion processes, and a working model (Fig. 8) was proposed (Costa *et al.* 2013a).

According to this model, on the apical dome of the PT, AGPs transduce external signals inward by establishing a network with transmembrane proteins, such as receptor kinases or C2 domain-containing proteins, in turn activating Ca^{2+} channels which are

known to trigger downstream intracellular events. During PT growth, AGPs are endocytosed, and either reprocessed or sent for degradation through multivesicular bodies. In the sub-apical zone, on the other hand, the calcium complexing, resultant from modification of the cell wall properties, impedes AGP contact with putative signalling molecules, making AGPs inactive. In many respects, this model resembles the ones proposed for Nicotiana by Lee *et al.* (2009) and de Graaf *et al.* (2003), mentioned earlier, to explain the internalization of tt AGPs inside the PTs while growing through the pistil tissues.

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CHAPTER 2

IMMUNOLOCALIZATION OF ARABINOGALACTAN PROTEINS (AGPS) IN REPRODUCTIVE STRUCTURES OF AN EARLY-DIVERGENT ANGIOSPERM, TRITHURIA (HYDATELLACEAE)

This chapter was based on:

Costa M, **Pereira AM**, Rudall PJ, Coimbra S. 2013. Immunolocalization of arabinogalactan proteins (AGPs) in reproductive structures of an early-divergent angiosperm, *Trithuria* (Hydatellaceae). Ann Bot. 111(2):183-190.

ABSTRACT

Trithuria is the sole genus of Hydatellaceae, a family of the early-divergent angiosperm lineage Nymphaeales (water-lilies). In this study different arabinogalactan protein (AGP) epitopes in *T. submersa* were evaluated in order to understand the diversity of these proteins and their functions in flowering plants. Immunolabelling of different AGPs and pectin epitopes in reproductive structures of *T. submersa* at the stage of early seed development was achieved by immunofluorescence of specific antibodies.

AGPs in Trithuria pistil tissues could be important as structural proteins and also as possible signalling molecules. Intense labelling was obtained with anti-AGP antibodies both in the anthers and in the intine wall, the latter associated with pollen tube emergence. AGPs could play a significant role in Trithuria reproduction, due to their specific presence in the pollen tube pathway. The results agree with labellings obtained for Arabidopsis and confirms the importance of AGPs in angiosperm reproductive structures as essential structural components and probably important signalling molecules.

INTRODUCTION

Arabinogalactan proteins (AGPs) belong to the hydroxyproline-rich glycoprotein superfamily with a very high level of type II arabinogalactan glycosylation (Showalter, 2001). AGPs are ubiquitous in plants and particularly abundant in cell walls, plasma membranes and extracellular secretions. Such ubiquitous presence implies that AGPs are vital components of the plant cell. Indeed, many studies have implicated AGPs in important biological phenomena such as cell expansion, cell division, cell death, seed germination, pollen-tube growth and guidance, and resistance to infection (Seifert and Roberts, 2007). However, the means by which these molecules function or interact with other cell components remain undetermined (Coimbra and Pereira, 2012). Pectins are other important and complex wall macromolecules consisting of homogalacturonans that can be methylesterified and acetylesterified, rhamnogalacturonans I, rhamnogalacturonans II and xylogalacturonans (Carpita and McCann, 2000). The cell-wall composition of early-divergent flowering plants is of great interest for understanding early evolution of angiosperm cell-wall structures. However, existing research in this field is limited. Among non-flowering land plants, AGPs were shown to be important for apical cell extension in the moss *Physcomitrella patens* (Lee *et al.* 2005). There have also been studies on green algae; for example, AGPs have been detected in *Micrasterias denticulata* (Eder *et al.* 2008), and a detailed study revealed several cell-wall polymers during antheridium development and spermatogenesis in *Chara corallina* (Domozych *et al.*, 2009). Localization of AGPs in tissues and cells can be demonstrated using specific monoclonal antibodies that bind to the structurally complex carbohydrate epitopes. The selective labelling obtained using these monoclonal antibodies in Arabidopsis and other plant species has shown that some AGPs are present as molecular markers during development and that they are probably related to important steps of the plant sexual reproductive cycle (Coimbra *et al.* 2007). The regulated expression and abundance of particular AGPs in the stigma, stylar transmitting tissue and pollen have led to the hypothesis that AGPs are important for plant pollen–pistil interactions (Coimbra and Pereira, 2012). AGPs are strong candidates for mediators of cell to cell communication, although the precise mechanism of AGP-mediated cell-wall signalling is unclear. Most, if not all, AGPs are anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor (Borner *et al.* 2003). AGPs can be released by cleavage of the GPI anchor using a specific phospholipase, in response to cellular signals. The high sugar content of

AGPs, together with the presence of the GPI anchor, makes these proteins major candidates for having key roles in reproductive processes, as for other eukaryotic systems (Coimbra and Pereira, 2012). However, to date, most studies of AGPs have focused on model organisms such as Arabidopsis. Comparative studies of a phylogenetically broad range of taxa are required to understand the diversity of these proteins and their functions.

The differences observed regarding the specific presence of selected epitopes during reproductive development will help us to address some fundamental questions related to the evolution and genetics of flowering. In the present work, we evaluate different AGP epitopes in the reproductive units of an early divergent angiosperm, *Trithuria submersa* (Hydatellaceae), which is the closest extant relative of the water-lilies (Saarela *et al.* 2007). Trithuria species represent useful models for comparative studies of this type, because (uniquely for early-divergent angiosperms) they are mostly rapidly growing annual plants that are readily cultivated *in vitro*. Due to its ready availability, recent studies have provided detailed morphological and molecular characterization of Trithuria, including studies on morphology and systematics (Hamann, 1998; Rudall *et al.* 2007, 2009a; Sokoloff *et al.* 2008a, b, 2011; Tratt *et al.* 2009; Iles *et al.* 2012), reproductive biology (Taylor *et al.* 2010; Prychid *et al.* 2011; Taylor and Williams, 2012), embryology, pollen morphology and seed development (Friedman, 2008; Remizowa *et al.* 2008; Rudall *et al.* 2008, 2009b; Tuckett *et al.* 2010a, b; Friedman *et al.* 2012). The present study will complement these studies of this phylogenetically significant taxon and broaden our knowledge of AGP structure and function in angiosperms.

MATERIALS & METHODS

Plant material and light microscopy

Reproductive units of *Trithuria submersa* grown at the Royal Botanic Gardens, Kew, were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffer (0.025 M, pH7, with one micro drop of Tween 80), placed under vacuum for 1 h and then at 48ºC overnight. After dehydration in a graded ethanol series, the material was embedded in LR White embedding resin (London Resin Company Ltd, London, UK). Thick sections (0.5 mm) were obtained with a Leica Reichert Supernova microtome placed on glass slides, and stained with a solution of 1% methylene blue for light microscopy, and 1% Lugol solution (Sigma-Aldrich 62650; St Louis, MO, USA) for starch staining.

Some slides were preserved unstained for immunolocalization of AGPs and pectin epitopes with monoclonal antibodies.

Immunolocalization of AGPs and pectins

A collection of monoclonal antibodies directed against glycosyl moieties specific to AGPs and pectins was provided by Prof. Paul Knox from the Centre for Plant Sciences, Faculty of Biological Sciences and University of Leeds, UK. The monoclonal antibodies recognizing AGP epitopes used were JIM8 (Pennell *et al.* 1991), JIM13 (Knox *et al.* 1991) and MAC207 (Pennell *et al.* 1989), and those recognizing pectin epitopes were JIM7 and JIM5 (Knox *et al.* 1990). LM6 (Willats *et al.* 1998) antibody can detect epitopes in AGPs and in pectins. The secondary antibody used was fluorescein isothiocyanate (FITC)-conjugated anti-rat IgG (Sigma-Aldrich F-1763).

Slides prepared for immunolocalization were circled with a Pap pen (Sigma-Aldrich Z672548), and treated as follows: 5 min in phosphate-buffered saline (PBS), pH 7.4, containing 5% non-fat dried milk (blocking solution), followed by incubation with primary antibody (diluted 1:5 in blocking solution), overnight and at 4°C. After washing with PBS, the sections were incubated with secondary antibody (diluted 1:100 in blocking solution) for 4 h in the dark, and then finally washed with PBS followed by distilled water. Slides were further stained with 0.01% calcofluor white (Fluorescent Brightener 28; Sigma-

Aldrich F3543, which is not removed) and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA).

Bright-field and fluorescence observations were made on a Leica DMLB epifluorescence microscope (objectives were Leica N-Plan, and filters were 365/445 nm for calcofluor and 470/525 nm for fluorescein stain). Images were captured with a ProgRes® MF cool (Jenoptik, Jena, Germany) in automatic exposure mode, and processed with ProgRes® CapturePro 2.8.8 software. Control experiments were performed omitting the incubation with the primary antibody (incubation with blocking solution only) and showed no unspecific staining. Confidence in specific antibody binding was reinforced by the different patterns of labelling obtained with the different monoclonal antibodies used.

Results

Reproductive units (RUs) of *Trithuria submersa* typically consist of numerous pistils surrounding one or two central stamens (Fig. 1A), all the fertile organs being enclosed by an involucre of bract-like phyllomes that resemble perianth organs. Pre-fertilized carpels were characterized here, which already contain the main seed storage tissue, the perisperm (e.g. Rudall *et al.* 2008). The ovule is bitegmic and the two cell-wall layers of each integument are clearly visible (Fig. 1B). At this stage of development, the micropyle is enclosed by the outer integument (Fig. 1C). A nucellar beak is also evident (Fig. 1C). For our study, the perisperm is the main tissue under investigation. As reported by Rudall *et al.* (2009b), the starchy perisperm develops precociously; the perisperm cells are arranged in groups whose walls start to break down and the nuclei clump together, resulting in a multinucleate appearance (Fig. 1B).

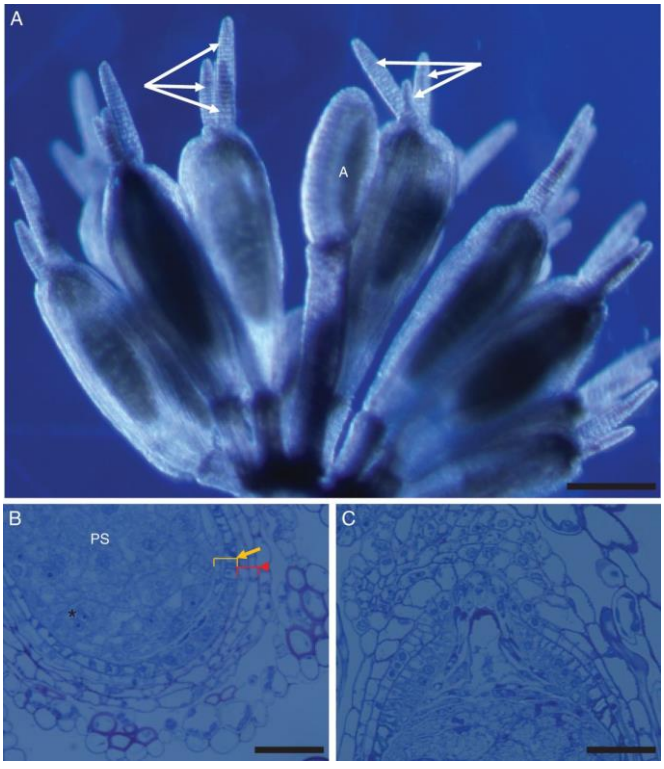

Figure 1 - *Trithuria submersa* reproductive structures. (A) *Trithuria submersa* reproductive unit, with a single stamen in the centre (labelled 'A') surrounded by several pistils, each with several long, multicellular uniseriate stigmatic hairs (arrows); scale bar = 200 µm. (B) Detail of lower part of ovule and carpel wall showing perisperm (PS) (*) with aggregated nuclei and two integuments, each with two cell layers (arrow, inner integument; arrowhead, outer integument); scale bar = 50 µm. (C) Detail of upper part of ovule and carpel wall showing the nucellar beak; scale bar = 50 µm.

Immunolocalization of AGPs and pectins in developing seeds of T. submersa

AGPs have been implicated in the sexual reproduction of several plant species and in Arabidopsis some AGPs were identified as molecular markers for gametophytic cell development (Coimbra *et al.* 2007). The monoclonal antibodies used that recognize AGP epitopes were JIM8, JIM13 and MAC207. For pectin epitopes, we used LM6, which recognizes a pentasaccharide of (1–5)-α-L-arabinans present in type I rhamnogalacturonan (Willats *et al.* 1998), although this is also present in some AGPs. Homogalacturonan epitopes were localized using JIM7 (Knox *et al.* 1990), which ligates to partially methyl-esterified homogalacturonans, and JIM5 (Knox *et al.* 1990), which has affinity for both partially methyl-esterified homogalacturonan and also non-esterified homogalacturonans. In pistils of *T. submersa*, the labelling obtained using LM6 was not uniform in all cell types (Fig. 2A). Cell walls are composed of cellulose microfibrils embedded in a polysaccharide matrix of hemicelluloses, pectins and other proteins, namely AGPs; the fact that the two integuments are not labelled with LM6 is significant (Fig. 2A). LM6 is present with high intensity in the embryo sac wall (Figs. 2A and 3A) and in the outer ovary wall (Fig. 3A). In contrast, labelling with JIM8 is relatively intense in the ovary wall and the outer integument (Fig. 2B), in the stigmatic hairs and on the nucellus tissue at the entrance to the embryo sac (Fig. 3C).

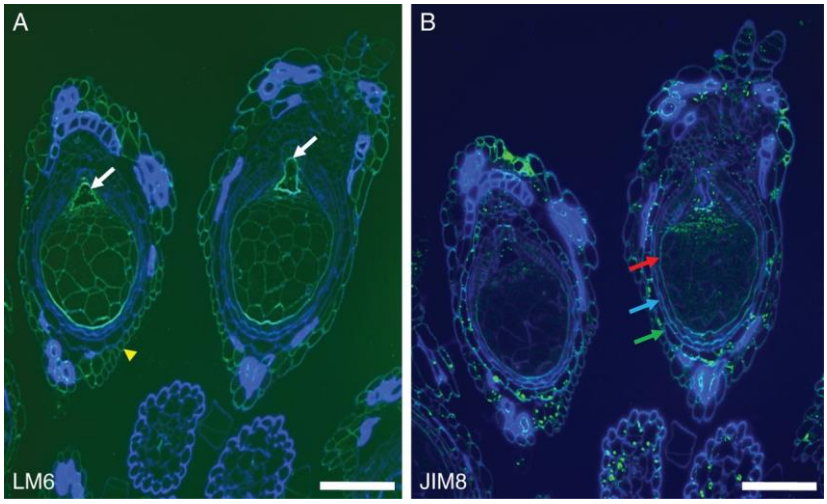

Figure 2 - Labelling with LM6 and JIM8. (A) LM6 labelling, showing a high intensity of labelling in the embryo sac wall (arrows) and the outer ovary wall (arrowhead). The perisperm cell walls are also labelled. (B) JIM8 labelling is relatively

intense in the ovary wall (red arrow), inner integument (blue arrow) and outer integument (green arrow). JIM8 labelling is also evident in some of the starch grains and in thickened cell walls. Scale bars = 200 µm.

The stigmatic hairs are strongly labelled with the anti-pectin LM6 and with the anti-AGPs JIM8 (Fig. 3B). LM2 antibodies also label intensely the stigmatic hairs and the ovary entrance (Fig. 3D) as well as the integuments and ovary wall (Fig. 3E). Curiously, when the antibody used was MAC207, the ovule and ovary cells were not labelled, but some secreted material present in the extracellular spaces was consistently labelled (Fig. 3F). Homogalacturonan epitopes were identified by JIM5 (Fig. 3G) and by JIM7 (Fig. 3H), although the labelling was not intense and mainly in the outer ovary cells wall and embryo sac wall.

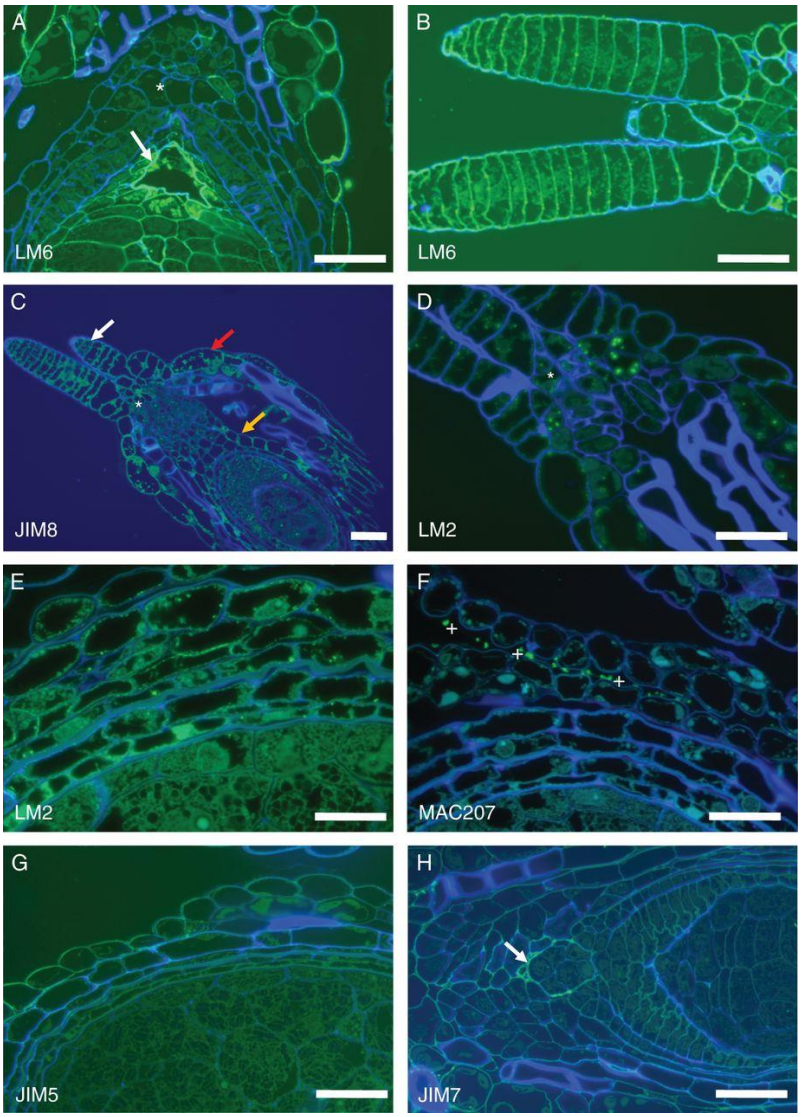

Figure 3 – Labelling with LM6, LM2, MAC207, JIM5, JIM7 and JIM8. (A) Details of LM6 labelling on the embryo sac wall (arrow) and nucellar beak (*). (B) Stigmatic hairs strongly labelled with LM6 which recognizes epitopes in pectins (i.e. the arabinan side-chains) and in AGPs. (C) JIM8 (as well as JIM13) labels the ovary wall (orange arrow), the outer integument (red arrow), the stigmatic hairs (white arrow)
and the region at the base of the stigmatic hairs (*). (D) LM2 antibodies intensely label the stigmatic hairs and the ovary entrance (*). (E) LM2-recognized epitopes are strongly present in the integuments and ovary wall. (F) Monoclonal antibody MAC207 consistently labels some secreted material present in the extracellular spaces (*), but not the ovule or the ovary cell walls. (G) JIM5 only labels the outer ovary wall while (H) JIM7 labels the embryo sac wall (arrow) the outer ovary wall and, very faintly, the perisperm. Scale bars: (A, B, D, G, H) = 50 µm; (C) = 100 µm; (E, F) = 20 µm.

Starch grains of T. submersa

Remarkably, we found that the two different types of starch grain present in different tissues of the same RU of *T. submersa* were differentially labelled by very similar anti-AGP antibodies such as JIM8 and JIM13. Specifically, JIM8 and LM2 both labelled equally; they are both absent from the perisperm starch grains (Figs. 4B, C), but are present in the outer layer of the starch grains of the outer integument, ovary wall, stamen filaments and bract-like phyllomes (Figs. 4B, E, F). Conversely, JIM13-recognized epitopes were present in the outer layer of the perisperm starch grains (Figs. 4A, D) and also present in the starch grains of the ovary wall, stamen filaments and bract-like phyllomes (Figs. 4A, G). In these plants, the amyloplasts may contain one or more starch grains; several grains of joint origin form a compound amyloplast, as evidenced by staining with lugol (Figs. 4H, I).

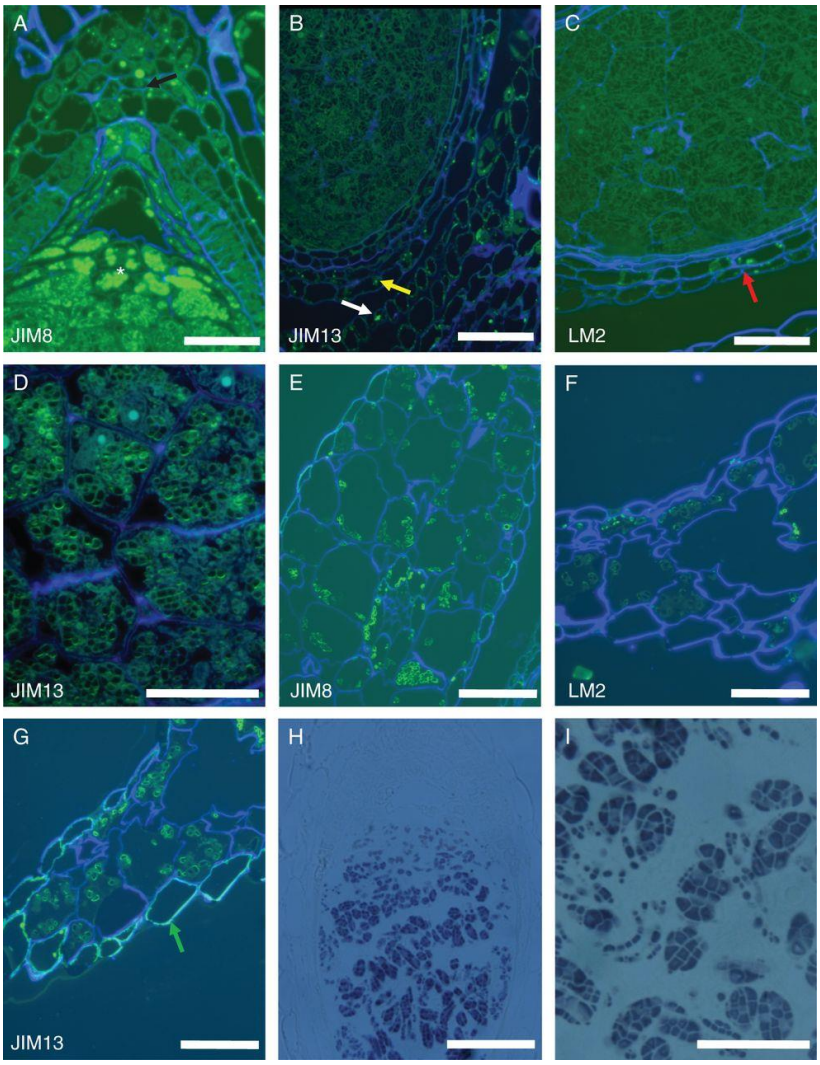

Figure 4 - Starch grains of different tissues in the same RU. (A) JIM13 labels the starch grains of the perisperm (*) and the outer integument (arrow). (B) JIM 8 and (C) LM2 both label only the starch grains present in the outer integument and ovary wall (arrows) and not the perisperm starch grains. (D) The labelling is associated with the outer layer of the perisperm starch grains. (E) JIM 8 and (F) LM2 both label equally the outer layer of the starch grains present in the bract-like phyllomes. (G) JIM13 labels intensely the outer layer of the starch grains present in the bract-like phyllomes and also the outer wall of the phyllome epidermal cells (arrow). (H) The amyloplasts are of the composed type, as evidenced by staining with lugol. (I) Higher magnification of the composed perisperm amyloplasts, stained with lugol. Scale bars: (A, B, E, F, G) = 50 µm; (C) = 20 µm; (D) = 10 µm; (H) = 100 µm.

Immunolocalization of AGPs and pectins in T. submersa anthers

Pollen of Trithuria is oblong or rounded and monosulcate (Remizowa *et al.* 2008). The aperture has a distinct margin and extends the full length of the pollen grain. It has already been shown that sometimes the aperture membrane bulges outwards (Prychid *et al.* 2011). Labelling with the antibodies JIM 8 and JIM 13 indicates some preferential presence for the AGPs identified by these antibodies in pollen grains, specifically at the intine wall and preferentially at the location where the pollen tube will emerge (Figs. 5A, B). At this stage of development, pollen is mature, the tapetum is already absent and the endothecium shows its typical wall thickenings. The entire intine wall is labelled with LM6 (Fig. 5C), rather than preferential labelling, as for the anti-AGP antibodies.

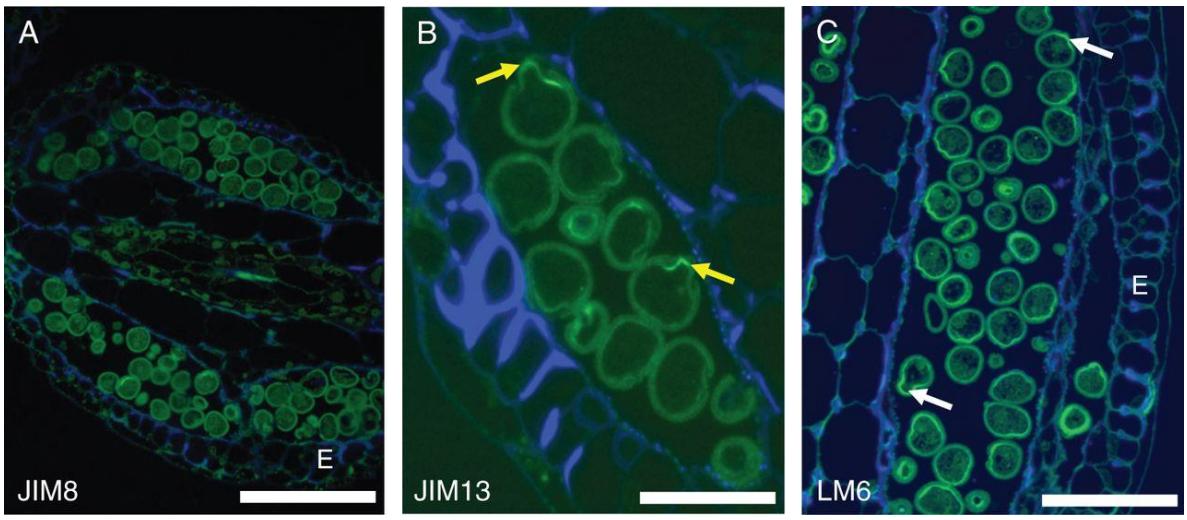

Figure 5 - Anther sections labelled with different antibodies. (A) JIM 8 and (B) JIM 13 preferential label the intine wall and especially at the aperture, where the pollen tube will emerge (arrows). (C) With LM6, the entire intine wall is labelled as expected, but the pectin wall is thicker at the aperture (arrows). E = endothecium. Scale bars: (A) = 50 µm, (B) = 10 µm, (C) = 20 µm.

Discussion

AGPs and pectins in pistils of T. submersa

Although the molecular mechanism of the action of AGPs remains unknown, mainly due to the difficulties posed by their complex glycoproteins, the selective labelling obtained here in mature RUs using the AGP monoclonal antibodies JIM8, JIM13, MAC207 and LM2 leads us to postulate that some AGPs are important for cell differentiation in *Trithuria submersa*.

AGPs are ubiquitous throughout the entire plant kingdom and have been shown to be involved in important developmental processes. They are probably related not only to the transition from a sporophytic type of development to a gametophytic one (Coimbra *et al.* 2007), but also in pollen development and pollen tube growth (Wu *et al.* 1995; Levitin *et al.* 2008; Coimbra *et al.* 2010). AGPs are important as cell-wall structural glycoproteins, but the fact that they are anchored to the cytoplasmic membrane by a GPI anchor makes them credible candidates for signalling molecules, as they are exposed to the extracellular matrix and can be cleaved by specific phospholipases.

A study of the stigmatic hairs and pollen tubes of Hydatellaceae has already shown that the uniseriate, multicellular stigmatic hairs of Trithuria label intensely with LM2, indicating that the AGP epitopes identified by this monoclonal antibody could be involved in pollen-tube attraction (Prychid *et al.* 2011). Here, we show that in addition to intense labelling in the stigmatic hairs, LM2 also labels AGP epitopes present at the bases of the hairs, in the micropylar nucellus and in the ovule integuments. The same result is true for the epitopes recognized by JIM8 and JIM13. These results agree with labelling obtained for Arabidopsis (Coimbra *et al.* 2007), and confirm the importance of AGPs in the reproductive structures as essential structural components and probably important signalling molecules.

Pectin is the most structurally complex family of polysaccharides in nature, making up to 35% of primary walls in eudicots and non-graminaceous monocots, 2–10 % of primary walls of grasses and other commelinid monocots, and up to 5% of walls in woody tissue (Mohnen, 2008). Surprisingly, we obtained differential labelling in the pistil tissues using LM6, JIM5 and JIM7, the monoclonal antibodies that label pectin epitopes. The embryo sac wall, the wall of the nutritive tissue and the ovary cell walls are strongly

labelled with LM6 while the integument cell walls are not labelled at all. This does not mean that these cells lack pectin in their walls, but that the pectins have different structures in different tissues of the pistil. The rhamnogalacturonan I (RG-I) type of pectin (the epitope recognized by LM6) is apparently absent from the integuments. Homogalacturonan epitopes labelled by JIM5 and JIM7 were evident only in the cell walls of the ovary and in the embryo sac wall. Labelling in the other cell types was faint or not present. Pectins are known to perform multiple functions in plant cells, starting with growth, development, morphogenesis, defence, cell–cell adhesion, wall structure, signalling, cell expansion, pollen tube growth, seed hydration, leaf abscission and fruit development (Mohnen, 2008). This is probably the reason that there are different wall constitutions in different tissues. The stigmatic hairs play an important role in adhesion for pollen tube growth, a function performed by AGPs and pectins. It is important to note that LM6 can also bind to AGPs (Lee *et al.* 2005).

As in Arabidopsis, no MAC207 labelling was present in the embryo sac wall of Trithuria (Coimbra *et al.* 2007). However, instead of being scattered throughout most cell types (as in Arabidopsis), this monoclonal antibody only labelled the extracellular secretions around the pistil in Trithuria. This difference could be consistent with the evolutionary distance between these two plant species, showing different constituents for equivalent organ tissues.

Starch grains of T. submersa

In leaves and stems, starch grains are stored transiently and provide for the immediate energy needs of the plant. In contrast, starch grains located in the ovules are storage starches used as long-term energy reserves. Starch grains contain two types of molecules composed of glucose residues, amylopectin and amylose. Amylopectin consists of ramified molecules consisting of glucose residues, while amylose is composed of linear glucose chains.

Unexpectedly, we found that AGP epitopes are associated with Trithuria starch grains. An even more unpredictable result was that the labelling of starch with anti-AGP antibodies was different according to the starch grain type. These results demonstrate that besides having different roles, these two types of starch grains are also different in composition.

AGPs and pectins in T. submersa anthers

The labelling obtained in the anthers with JIM8 and JIM13 was also significant. It is known that these two monoclonal antibodies specifically label the gametophytic cells in Arabidopsis (Coimbra *et al.* 2007). The labelling obtained for Trithuria was intense, but located in the intine wall at the aperture, associated with future pollen tube emergence. In many plants, AGPs are present in the pollen-tube wall (Mollet *et al.* 2002; Pereira *et al.* 2006; Qin *et al.* 2007; Dardelle *et al.* 2010) and they are probably involved in signalling during pollen tube growth, but it is interesting to note that the AGP epitopes present are different in these diverse plant species. With these results we can propose that AGPs are important surface molecules, involved in pollen tube growth and most probably also in pollen–pistil interactions.

Conclusions

In conclusion, the specific and strong labelling obtained with monoclonal anti-AGP antibodies demonstrate the importance of these proteins in Trithuria, a plant that is phylogenetically placed close to the root of extant angiosperms. Although important for plant reproduction due to the specific presence in the pollen tube pathway, AGPs also show the evolutionary divergence related to the cell-wall composition of specific cell types, such as pollen tubes, ovule tissues and style, probably related to the evolution of reproductive strategies.

Acknowledgments

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CHAPTER 3

DIFFERENTIAL EXPRESSION PATTERNS OF ARABINOGALACTAN PROTEINS IN *ARABIDOPSIS THALIANA* REPRODUCTIVE TISSUES

This chapter was based on:

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ABSTRACT

Arabinogalactan proteins (AGPs) are heavily glycosylated proteins existing in all members of the plant kingdom and are differentially distributed through distinctive developmental stages. Here, we showed the individual distributions of specific Arabidopsis AGPs: AGP1, AGP9, AGP12, AGP15, and AGP23, throughout reproductive tissues and indicated their possible roles in several reproductive processes. *AGP* genes specifically expressed in female tissues were identified using available microarray data. This selection was confirmed by promoter analysis using multiple green fluorescent protein fusions to a nuclear localization signal, β-glucuronidase fusions, and *in situ* hybridization as approaches to confirm the expression patterns of the AGPs. Promoter analysis allowed the detection of a specific and differential presence of these proteins along the pathway followed by the pollen tube during its journey to reach the egg and the central cell inside the embryo sac. *AGP1* was expressed in the stigma, style, transmitting tract, and the chalazal and funiculus tissues of the ovules. *AGP9* was present along the vasculature of the reproductive tissues and *AGP12* was expressed in the stigmatic cells, chalazal and funiculus cells of the ovules, and in the septum. *AGP15* was expressed in all pistil tissues, except in the transmitting tract, while *AGP23* was specific to the pollen grain and pollen tube. The expression pattern of these *AGPs* provides new evidence for the detection of a subset of specific AGPs involved in plant reproductive processes, being of significance for this field of study. AGPs are prominent candidates for male–female communication during reproduction.

INTRODUCTION

All flowering plants share a common characteristic that distinguishes them from all other organisms that reproduce sexually: double fertilization (Raghavan, 2003). During this process, two male sperm cells are delivered to the female gametophyte—the embryo sac—where one fuses with the egg and the other fuses with the central cell, giving rise to the embryo and the endosperm, respectively (Russell, 1992). In order for the sperm cells to be delivered into the embryo sac, several events need to occur, which implicates tightly regulated interactions between the female sporophytic tissues and the male gametophyte. Once the pollen grain is in contact with the stigmatic cells, it germinates, producing the pollen tube (Kandasamy *et al.* 1994), which will deliver the two sperm cells to their final destination (Faure *et al.* 2002; Dresselhaus and Franklin-Tong, 2013). In the majority of seed plants, the pollen tube grows through the stigmatic cells, into the style, and across the extracellular matrix of the transmitting tissue in a very precise way, never losing its focus, to reach the embryo sac. Once at the placenta, it makes a quick turn and grows on the surface of the funiculus until reaching the ovule opening, the micropyle (Hülskamp *et al.* 1995). After growing through the micropyle, the pollen tube enters the female gametophyte, interacts with one of the two synergid cells and bursts, releasing the two sperm cells that will fuse with the central and the egg cell, ultimately giving rise to the seed and assuring the perpetuation of the next generation (Johnson and Preuss, 2002; Lord and Russell, 2002; Raghavan, 2003; Berger *et al.* 2008; Sprunck, 2010; Palanivelu and Tsukamoto, 2012).

During the course of all these processes, numerous cell–cell communication events must take place between different cell types. Mainly, recognition signals and attracting signals have to be sent and perceived by the female tissues and the male tissues of the plant, and vice versa, in order for a successful fertilization to occur (Dresselhaus, 2006). To date, despite all the efforts carried out in this field of study, little information is available about which molecules function as signalling or receptor molecules.

Arabinogalactan proteins (AGPs) constitute a large family of hydroxyproline-rich proteins that are highly glycosylated and structurally complex (Showalter, 2001). AGPs are widely distributed in the plant kingdom, being ubiquitously present in land plants and also in the Bryophyta Physcomitrella patens (Lee *et al.* 2005; Fu *et al.* 2007) and in all Hepatophyta (Basile *et al.* 1989), including basal angiosperms (Costa *et al.* 2013b) and many algae, indicating an ancient origin for these proteins (Popper *et al.* 2011).

They are found in distinct developmental stages and cell, tissue, and organ types, being mostly abundant in cell walls, plasma membranes, and extracellular secretions (Majewska-Sawka and Nothnagel, 2000). AGPs are typically divided into four subgroups according to their polypeptide core characteristics: the classical AGPs, which possess an N-terminal signal peptide that is removed in the mature protein, a proline/hydroxyproline-rich domain and a C-terminal signal for the addition of a glycosylphophatidylinositol (GPI) anchor; the arabinogalactan (AG) peptides, structurally similar to the classical AGPs but with a smaller protein backbone, consisting of 10–13 aa; the lysine-rich AGPs, with one or more lysine domains; and the fasciclin-like AGPs (FLAs) with one or more fasciclin-like domains in their polypeptide core (Schultz *et al*. 2002; Johnson *et al.* 2003).

AGPs have been implicated in many important processes for plant development and growth, such as cell expansion, proliferation and differentiation, cell–cell recognition, somatic embryogenesis, pollen tube growth, PCD, seed germination, and resistance to infection (Majewska-Sawka and Nothnagel, 2000). Most AGPs are predicted to be anchored to the membrane by a GPI anchor (Borner *et al.* 2002; Schultz *et al.* 2004), which provides a way for the AGPs to function as signalling molecules. After comparisons with GPI-anchored proteins from animal cells, two mechanisms were proposed for AGP-mediated signalling: the first consisted of the cleavage of the GPI anchor by specific phospholipases (C and D) that would release the glycoprotein into the extracellular matrix, making it able to act as a signal itself or to be subject to further processing, generating different signals; the other mechanism proposed that AGPs could interact with other proteins and activate downstream signal transduction pathways (Gaspar *et al.* 2001; Schultz *et al.* 2004). Besides the hint given by the presence of the GPI anchor, implying a signalling role for these proteins, the prominent carbohydrate content surrounding the core protein also led to some assumptions about their involvement in signalling mechanisms. The importance of sugars as signalling molecules in plants is well known, and, according to some authors, the varied carbohydrate moieties of AGPs might be released via cleavage by specific enzymes (Showalter, 2001). The generated oligosaccharides might function as signalling molecules by binding to specific membrane receptors and activating specific signal transduction systems (Showalter, 2001). The fact that AGPs can act as chitinase substrates, being able to stimulate somatic embryogenesis, reinforces this hypothesis, although it has not yet been demonstrated whether this is an effect of the released oligosaccharides or the modified AGP (Van Hengel *et al.* 2001).

AGPs have long been suggested to play important roles in sexual plant reproduction. Earlier studies have shown the developmentally regulated enrichment of

AGPs in the extracellular matrix of the transmitting tract of several species such as *Gladiolus gandavensis*, *Lilium longiflorum*, *Nicotiana alata*, and *Lycopersicon peruvianum* (Hoggart and Clarke, 1984; Sedgley *et al.* 1985; Webb and Williams, 1988; Gane *et al.* 1995). AGPs have also been implicated in pollen tube growth from the stigma to the ovules in *Amaranthus hypochondriacus*, *Actinidia deliciosa*, *Catharanthus roseus*, and *Nicotiana tabacum* (Coimbra and Salema, 1997; Cheung *et al.* 1995; Coimbra and Duarte, 2003). These studies were carried out using the β-glycosyl Yariv reagent that binds specifically to AGPs, precipitating them (Yariv *et al.* 1967), or using monoclonal antibodies that identify only the glycosidic epitopes of AGPs (Pennell *et al.* 1989, 1991; Knox *et al.* 1991). These two approaches have given us information about the distribution and localization of AGPs (Coimbra *et al.* 2007) and clues about their possible roles (Gao and Showalter, 2002; Sardar *et al.* 2006), although they allow only the detection of general AGPs and not a specific AGP. The recent discovery that the Yariv reagent binds specifically to the β-1,3-galacto-oligosaccharides of AGPs (Kitazawa *et al.* 2013) may bring new insights to the possible mode of action of AGP oligosaccharides as signalling molecules. It will be interesting to check whether this particular oligosaccharide is important for many of the physiological processes impaired when Yariv was used in different studies, or if Yariv only hampers the ability of AGPs to function by precipitating them.

Here, we report the use of several constructs to explore the tissue and cell-specific promoter activity of specific AGPs. We have focused on those AGPs that are particularly present along the pollen tube pathway and other female reproductive tissues, according to the available microarray data. With this, we aimed to complement work that has already been done by our group describing AGPs as molecular markers of different stages of Arabidopsis sexual reproductive processes (Coimbra *et al.* 2007).

MATERIALS & METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana (L.) Heynh. seeds, ecotype Columbia were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Plants were sown on soil, kept for two days at 4°C in the dark to induce stratification, and afterwards, they were grown at 22°C under a short day photoperiod (9h/15h light/dark cycles) for four weeks, followed by a long day photoperiod (16h/8h light/dark cycles) to induce flowering, with 60% relative humidity. For PAT (Phosphinotricin-Acetyltransferase) selection the seedlings were sprayed with 200 mg l^{-1} glufosinate ammonium (BASTA®; Bayer Crop Science) supplemented with 0.1% Tween 20 for three or four times, every two days, during a ten day period.

Constructs generation and plant transformation

Genomic regions corresponding to the promoters of five AGPs: *AGP1*, *AGP9*, *AGP12*, *AGP15*, *AGP23* were amplified using Phusion DNA polymerase (Thermo Scientific), with the primer pairs described in Supplemental Table 1 (Appendix 1). The promoter regions were always amplified from the end of the UTR of the most proximal gene upstream of the respective AGP until its own start codon. For the genes with promoter regions with more than 3000 bp, genomic fragments of about 3000 – 3300 bp positioned upstream of the start codon of the *AGP* of interest were amplified. The PCR products were cloned into pENTR™/D-TOPO (Invitrogen). The resulting promoter fragments were subsequently transferred into a Gateway-compatible version (Zheng *et al*. 2011) of the pGreenII-based vector NLS:3GFP:NOSt (Takada and Jürgens, 2007), termed pGII_GW:NLS:3GFP:NOSt. For *AGP1*, *AGP15* and *AGP23* GUS constructs, the respective promoter fragments were cloned into the binary vector pBGWFS7 (Karimi *et al.* 2002). All constructs were confirmed by DNA sequencing. The pGreenII-based expression vectors were introduced into *Agrobacterium tumefaciens* GV3101 harbouring the pGreenII helper plasmid, pSOUP. All the others expression vectors were delivered into *Agrobacterium tumefaciens* GV3101 (pMP90RK). All of them were then used to transform *Arabidopsis thaliana* (Col-0) by the floral dip method (Clough and Bent, 1998).

Preparation of plant material for microscopy

Pistils kept in 50 mM sodium phosphate buffer (pH 7.5) were dissected under a stereomicroscope (Model C-DSD230, Nikon) using hypodermic needles (0.4 x 20 mm; Braun). The opened carpels and the ovules that remained attached to the septum were maintained in mounting medium and covered with a cover slip.

Confocal Laser Scanning Microscopy (CLSM)

A Zeiss Axiovert 200M inverted microscope equipped with a confocal laser-scanning module (LSM 510 META) was used for CLSM. GFP was excited by 488 nm and detected with a BP 505-550 filter. Optical sections were generally between 0.40 and 0.50 µm each, observed at x20, x40 or x63 magnifications. Histology mounting medium Fluoroshield™ with 4',6-diamidino-2-phenylindole (DAPI, Sigma) was used in order to detect the nuclei in the pollen grains. Images were captured and processed using an AxioCam HRc camera, Zeiss LSM 510 META software and a Zeiss LSM image browser version 3.5.0.359.

Detection of GUS activity

GUS assays were performed on inflorescences as described in Liljegren *et al.* (2000), overnight. After chemical GUS detection, the samples were incubated in clearing solution [160g of chloral hydrate (Sigma-Aldrich), 100mL of water, and 50mL of glycerol] and incubated at 4ºC overnight. The next day, inflorescences were dissected under a stereomicroscope (model C-DSD230, Nikon), and observed under a microscope. A Zeiss AxioImager AZ microscope equipped with differential interference contrast (DIC) optics was used. Images were captured with a Zeiss Axiocam MRc3 camera using Zen Imaging Software.

Phylogenetic Analysis

To generate a phylogenetic tree for the AGP genes, the amino acid sequences of AGP-coding sequences were aligned using Clustal W (Thompson *et al.* 1994) and manually edited using Jalview to reduce gaps (Clamp *et al.* 2004). A neighbor-joining (NJ) (Saitou *et al.* 1987) tree was generated using the MEGA4 program (Tamura *et al.* 2007).

The bootstrap values were obtained by 10,000 repetitions. Simultaneously, a maximum parsimony tree was generated using the same AGP amino acid sequences, and a NJ tree was also produced using only the three most conserved blocks of AGP amino acid sequences.

Preparation of plant material for RNA extraction

Arabidopsis pistils from wild-type plants were emasculated 1 day before anthesis and collected 2 days after the emasculation procedure. Pollen from wild-type Arabidopsis recently opened flowers was collected according to Costa *et al.* (2013a). Arabidopsis seeds were sown in half strength Murashige and Skoog medium, complemented with 0.7% agar. Agar plates were kept for 2 days at 4°C in the dark, to induce stratification, and subsequently they were transferred to a growth chamber at 22°C under a long day regime (16h light/8h dark), with irradiance of 130 µmol m^{-2} s^{-1} and 60% relative humidity. Seedlings were collected 4-5 days after germination.

RNA extraction, cDNA synthesis and Real Time RT-PCR

Total RNA from emasculated pistils, pollen and seedlings was extracted using PureZol™ RNA Isolation Reagent (Bio-Rad) following the manufacter's instructions. DNA was removed by a DNase (Thermo Scientific) treatment. The isolated RNA samples were reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and oligo$(dT)_{18}$ primers to initiate the reactions, following the manufacter's intructions.

cDNA was amplified using the SSoFast™ SYBR® Green Supermix on an iQ5™ Real-Time PCR Detection System (Bio-Rad) using the primers listed in Supplemental Table 2 (Appendix 1). Real-time RT-PCRs were run in duplicates. After 3 min at 95°C followed by a 10 s denaturation step at 95°C, samples were run for 40 cycles of 10 s at 95°C and 30 s at 60°C. After each run, a dissociation curve was acquired to check for amplification specificity by heating the samples from 60 to 95°C. Serial dilutions of pure genomic DNA from Arabidopsis ecotype Columbia were used to set up a calibration curve, which was used to quantify plant DNA in each sample. At the end of the PCR cycles, data were analysed with iQ5 2.0, Standard Edition Optical System Software v2.0.148.060623 (Bio-Rad).

Fluorescence in situ hybridization (FISH)

Genomic DNA was obtained as described by Edwards *et al.* (1991) and used to amplify the *in situ* sense and anti-sense probes for *AGP1* and *AGP12* using the following primers: AGP1-F 5´-CAAAAACACTCCCAAACCAAA-3´, AGP1-R 5´-CTTCAGTCGGAGAATCGG-3', AGP12-F 5´-CACAACTCATCATTCGCACCAAAG-3´ and AGP12-R 5´-GCATCGGAAGTAGGACTTGG-3´. The amplified fragments were cloned in pGEMT-Easy (Promega). DIG-RNA probes were generated by *in vitro* transcription using the DIG-RNA labelling kit (Roche). The dissected pistils were permeabilized by first dehydrating in a methanol series of increasing concentration and then rehydrating in a methanol series of decreasing concentration. The pistils where then treated with 2% cellulase (Onozuka R-10) for 1 h, and then washed and dried. RNA/RNA FISH was performed as described by Testillano and Risueño (2009); using DIG-RNA probes diluted 1:50 in hybridization buffer at 50°C overnight. Post-hybridization washes were performed in 4× SSC, 2× SSC, and 0.1× SSC. The hybridization signal was detected by incubation with mouse anti-DIG antibodies (1:5000 in 1% BSA; Sigma) for 90 min, followed by incubation with Alexa Fluor 488-conjugated anti-mouse antibody (diluted 1:25 in PBS; Molecular probes) for 45 min. After washing in PBS, sections were counterstained with DAPI, mounted in Mowiol, and observed by confocal microscopy. Controls were performed using the sense probes.

RESULTS

Phylogenetic analysis and AGPs distribution across the genome

An alignment of full-length predicted AGP proteins was generated using Clustal W (Thompson *et al.* 1994) and then manually refined (Fig. 1A). In this study, 13 classical AGPs (AGP1, AGP2, AGP3, AGP4, AGP5, AGP6, AGP7, AGP9, AGP10, AGP11, AGP25, AGP26, AGP27), 10 AG peptides (AGP12, AGP13, AGP14, AGP15, AGP16, AGP20, AGP21, AGP22, AGP23, AGP24) and three lysine-rich AGPs (AGP17, AGP18, AGP19) were considered. For this analysis, only four Fasciclin-Like AGPs (FLAs) were used: FLA18, FLA16, FLA17 and FLA15. These FLAs were chosen randomly and included in the analyses only as outgroup, since they are particularly different from the rest of the family and considered to be chimeric AGPs (Showalter *et al.* 2010). The phylogenetic distribution of the selected AGP sequences partially supported the four sub-groups of AGPs proposed by previous studies (Schultz *et al.* 2002; Johnson *et al.* 2003). The alignments showed a high level of similarity between the predicted amino acid sequences of AGP15, an AG peptide, and AGP1, a classical AGP, as well as the inclusion of the three lysine-rich AGPs in the same branch as the classical AGPs, not supporting the AGPs classification currently in use. As expected, the FLAs used in this study aligned together and independently from the other AGPs as a sub-group, but were still related to the classical AGP25, AGP26 and AGP27. Comparing the NJ tree generated using the AGPs complete amino acid sequeneces with the maximum parsimony tree and the NJ tree generated using only the three conserved blocks of AGP amino acid sequences (Supplemental Figs. 1 and 2), we could see almost no difference among them. All the main blocks of closely related AGPs remained grouped together in the three different trees, reinforcing the strength of this analysis. These two trees were generated using the most conserved regions revealed by the AGP multiple alignments (Supplemental Fig. 3). Looking at the AGP gene distribution along the different five Arabidopsis chromosomes (Fig. 1 B), there was no evidence of clustering of any specific group of closely related AGP genes, or any specific class of AGPs. They seemed to be randomly distributed across the different Arabidopsis chromosomes.

AGPs gene expression

As a first approach, data from microarray experiments available from on-line databases such as Genevestigator (http://genevestigator.ethz.ch; Zimmermann *et al*. 2004).

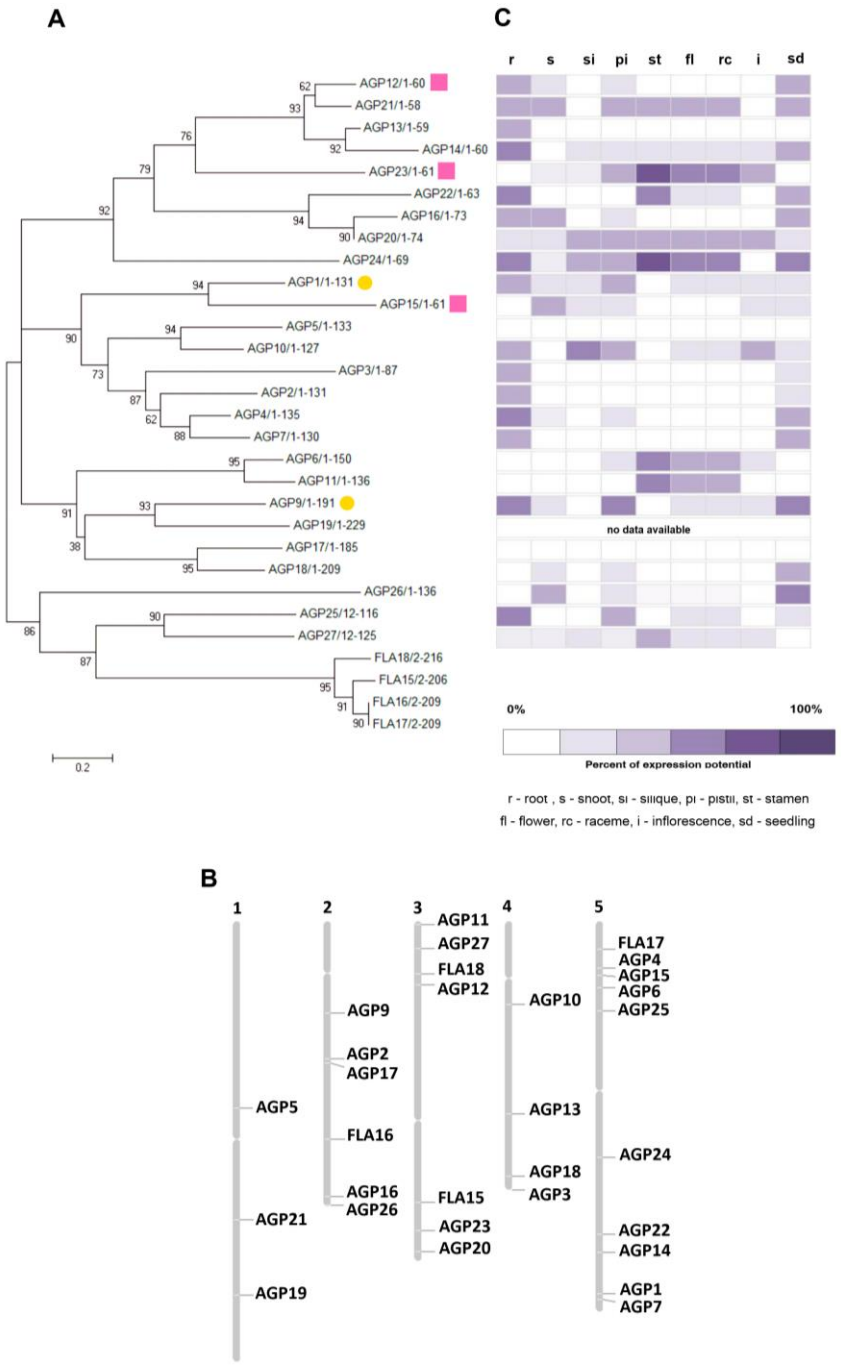

Figure 1 - The AGP protein family, gene expression, and *AGP* gene localization in *A. thaliana*. (A) Phylogenetic analysis of the AGP family in *A. thaliana*. To generate the phylogenetic tree for AGPs, all the amino acid sequences of AGP-coding sequences were aligned using Clustal W and manually edited using Jalview to reduce gaps. A NJ tree was generated using the MEGA4 program. The optimal tree with the sum of branch length=14.47033254 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (10 000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site (bar). The analysis involved 30 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of

241 positions in the final dataset. AGPs selected for further analysis are indicated by a yellow circle (classical AGPs) and a pink square (AG peptides). (B) The 26 *AGP* and four *FLA* genes were localized on the Arabidopsis chromosomes using the Chromosome Map Tool available at The Arabidopsis Information Resource (TAIR: http://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp). (C) Gene expression patterns for the 26 *AGP* and four *FLA* genes obtained using Genevestigator.

and the Arabidopsis eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter et al. 2007) were used to evaluate the distinct levels of AGPs genes expression throughout the different plant tissues (Fig. 1C, only Genevestigator data are shown). Eleven *AGPs* were selected for further analysis: *AGP1*, *AGP4*, *AGP7*, *AGP9*, *AGP10*, *AGP12*, *AGP15*, *AGP16*, *AGP23*, *AGP25* and *AGP26*, most of them based on the presence of their transcripts in pistil tissues and their absence in stamen tissues. In the case of *AGP7*, although it did not show this pattern of expression, it was selected anyway, based on its predicted high level of amino acid sequence similarity with *AGP4*. *AGP23* was selected as a negative control, since eFP Browser and the literature data (Costa *et al.* 2013c; Nguema-Ona *et al.* 2013) indicated that it is expressed only in pollen. However, the Genevestigator data also indicated poor expression in the female tissues. To check the differences between *AGP* gene expression levels in these tissues and to validate the microarray-based information, a real-time RT-PCR was performed using emasculated pistils, pollen from flowers at anthesis (stage 13 according to Smyth *et al.* 1990) and seedlings cDNA. The results confirmed the microarray data initially considered (Fig. 2).

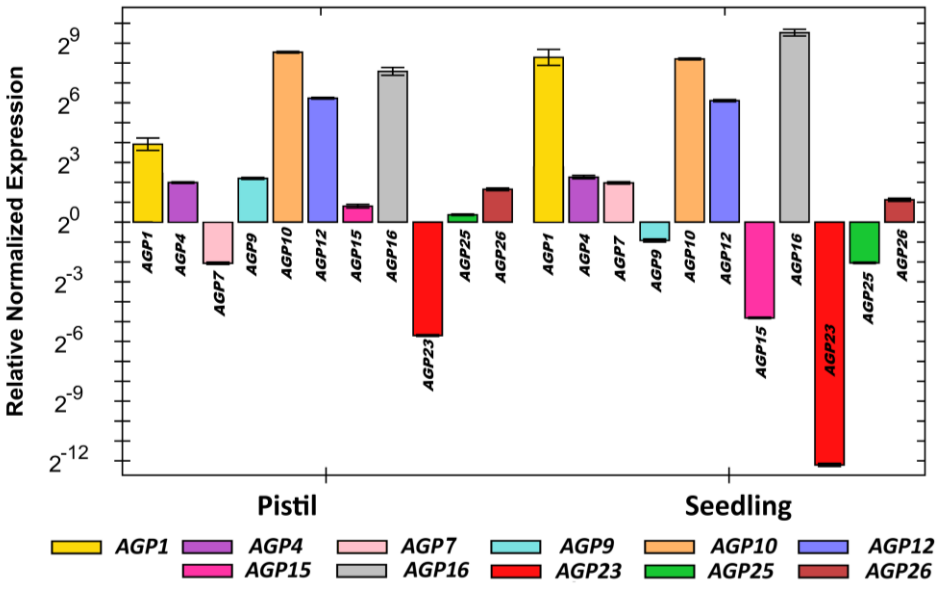

Figure 2 - Quantitative PCR relative expression levels of the selected *AGP* mRNA transcripts in emasculated pistils, pollen, and seedlings of wild-type Arabidopsis plants. The pollen was collected from anthers at stage 12 of flower development according to Smyth *et al.* (1990). *AGP* transcript levels were normalized to *ACT8* and *RUB1* reference gene levels, and are presented relative to the pollen transcript levels. Each bar represents an average of two independent reactions and technical replicates.

These analyses confirmed the good quality of the microarray data. In this work the *AGP* transcript levels were normalized to *ACT8* and *RUB1* reference gene levels, and are presented relative to the pollen transcript levels, since the main goal was to determine the *AGP* genes that are expressed more in the female tissues than in the pollen. *AGP7* and *AGP23* are down-regulated in the pistil tissues when compared to their expression in pollen, while all the others *AGPs* were up-regulated. *AGP10*, *AGP12* and *AGP16* were the genes that revealed a higher level of overexpression when compared to their expression in pollen. *AGP1*, *AGP4*, *AGP15*, *AGP25* and *AGP26* were revealed to be up-regulated in the pistils, comparing to pollen, but not at such high levels as the genes *AGP10*, *AGP12* and *AGP16*. From this group of up-regulated *AGPs*, *AGP1*, *AGP9*, *AGP12* and *AGP15* were selected for further analyses.

Plasmid construction and expression in A. thaliana

To improve the visualization and to avoid diffuse fluorescent signals in the detection of the promoter activities, the reporter gene *NLS:3GFP* was used (Takada and Jürgens, 2007). This consists of the simian virus 40 nuclear localization signal (NLS) and three tandem enhanced GFP (3×EGFP) sequences (Fig. 3A). The fluorescent signal should be then targeted to the nuclei, thereby enhancing the sensitivity of the GFP signal. In all the transgenic *A. thaliana* plants bearing the different p*AGP*:NLS:3GFP constructs, the GFP reporter expression was limited to the nuclei as expected, as shown in Fig. 3B–M.

AGPs differential expression pattern in A. thaliana reproductive tissues

The *AGP* promoters selected for this study allowed us to detect the different patterns of expression of these proteins in the female reproductive tissues. All the flowers analysed were between stages 12 and 13 according to Smyth *et al.* (1990). GFP expression driven by the *AGP1* promoter was strong in the style tissues (Fig. 3B), septum (Fig. 3C), transmitting tract (Figs. 3D), funiculus that attaches the ovules to the placenta (Figs. 3C, D), and chalazal region of the ovules (Fig. 3D). Weaker GFP expression was detected in the stigmatic cells (Fig. 3B) and the integuments of the ovule (Fig. 3D). The *AGP12* promoter guided the expression of GFP strongly to the stigmatic cells (Fig. 3E) and the chalazal pole of the ovules (Fig. 3F).

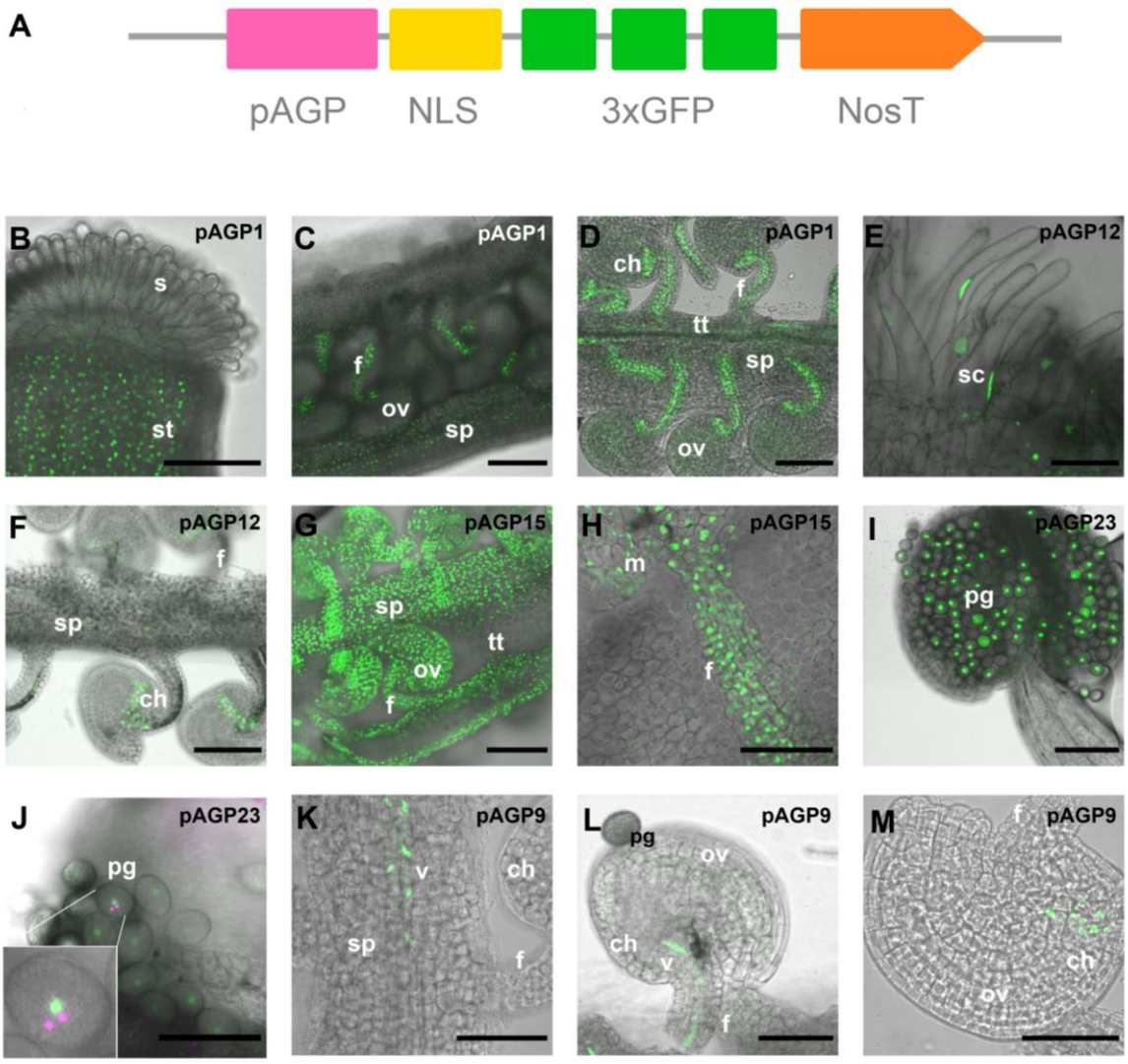

Figure 3 - Schematic representation of the expression cassette used in this study, and the resulting GFP signal shown in Arabidopsis reproductive tissues. (A) Expression cassette showing the relative position of the promoter sequence (p*AGP*), NLS, a fusion of three GFPs (3×GFP), and the terminator Nos (NosT). (B–D) NLS:3GFP expression driven by the *AGP1* promoter in the style tissues (B), the opened pistil, and the funiculus and septum tissues (C), and seen in more detail in the transmitting tissue, funiculus, and the chalazal pole of the ovule (D). (E–F) NLS:3GFP expression under the control of the *AGP12* promoter was observed in the stigmatic cells (E) and in the chalazal pole of the ovule (F). (G–H) NLS:3GFP expression driven by the *AGP15* promoter was detected in the ovule integuments, funiculus, and septum, but absent from the transmitting tissue (G). In (H) the GFP signal is seen in more detail in the nuclei of the funiculus. (I, J) NLS:3GFP under the control of the *AGP23* promoter is absent in all the sporophytic tissues (I), with its expression restricted to the pollen grain, and, as can be seen in the insert in (J), DAPI staining (here in magenta) revealed this expression to be limited to the vegetative cell of the pollen grain; DAPI-stained germinative nuclei are visible (white arrowheads). (K–M) NLS:3GFP signals expressed by the *AGP9* promoter. Signals were observed in the vascular bundle of the transmitting tract (K) and funiculus (L) as well as in the chalazal pole of the ovule (M). All the flowers used in these observations were at stages 12 and 13 according to Smyth *et al.* (1990). ch, Chalaza; f, funiculus; m, micropyle region of the ovule; ov, ovule; pg, pollen grain; s, stigma; sc, stigmatic cell; sp, septum; st, style; tt, transmitting tract; v, vascultature. Bars, 100 µm (B–G, I); 50 µm (H, K–M); 20 µm (J).

Very weak GFP expression was observed along the internal tissues of the funiculus and septum (Fig. 3F). Plants transgenic for the p*AGP15*:NLS:3GFP expression cassette exhibited GFP expression in all the female reproductive tissues, except in the transmitting tract cells (Figs. 3G, H). The *AGP23* promoter drove GFP expression specifically into the vegetative cell of the pollen grains (Figs. 3I, J). This was clarified by the DAPI staining of the pollen grains, showing that the GFP signal was present only in the nucleus of the vegetative cell and not in the generative cell nuclei, where there was only DAPI staining without any green signal. The *AGP9* promoter led to the expression of GFP in the vascular tissues of the pistil transmitting tract, the septum (Fig 3K), and the funiculus (Fig. 3L), exhibiting very weak expression in the chalazal pole of the ovules (Fig. 3M).

At the same time, p*AGP*:GUS constructs were also analysed for three *AGPs*: *AGP1*, *AGP15*, and *AGP23*. For the p*AGP1*:GUS fusion-expressing plants, a low GUS activity was observed in the stigmatic cells, while a higher GUS activity was detected in the septum, transmitting tract, funiculus, chalaza, and ovule integument cells (Figs. 4A, B). Regarding the plants expressing GUS under the control of the *AGP15* promoter, a high GUS activity was detected in almost all the tissues of the pistil, except in the transmitting tract (Figs. 4C, D). As well as the plants expressing the three GFP molecules under the control of the *AGP23* promoter, the Arabidopsis plants bearing GUS under the control of this same promoter showed a very specific and high GUS activity in the pollen (Figs. 4E, F).

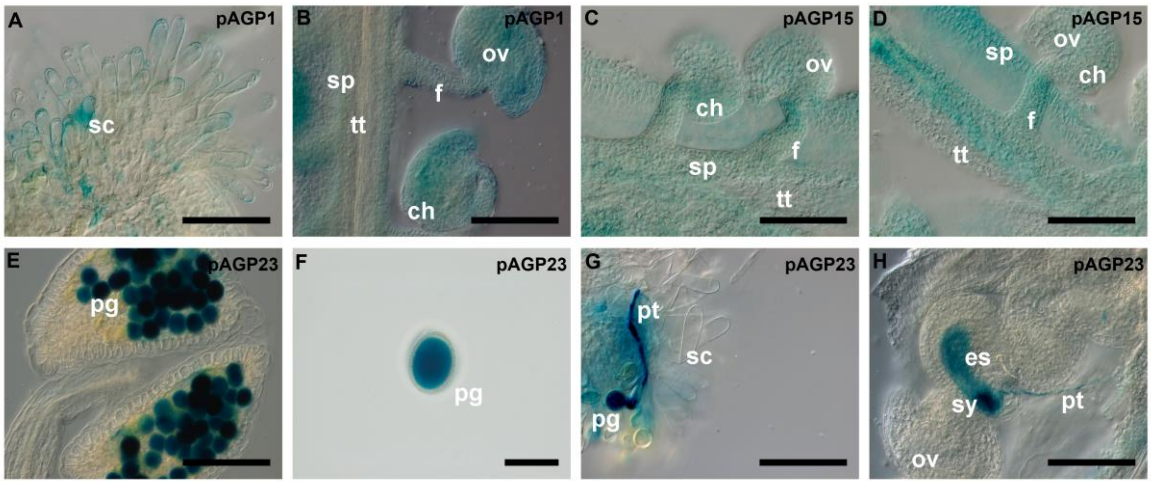

Figure 4 - Histochemical localization of GUS activity in transgenic Arabidopsis reproductive tissues expressing the p*AGP*:GUS fusion genes. (A, B) GUS activity driven by the *AGP1* promoter is detected in the stigmatic cells (A) and the transmitting tract, funiculus, and integument cells (B). (C, D) GUS activity driven by the *AGP15* promoter observed in the ovule integuments, funiculus, and septum cells. (E–H) Strong GUS activity driven by the *AGP23* promoter was identified

inside pollen grains (E) and (F) and the growing pollen tube (G). Upon fertilization, inside the embryo sac, a strong staining is observed at the local where the pollen tube bursts (H), followed by a weak staining that spread inside the whole embryo sac (H). Flowers of stages 12 and 13 (Smyth *et al.* 1990) were used in this study. ch, Chalaza; es, embryo sac; f, funiculus; ov, ovule; pg, pollen grain; pt, pollen tube; sc, stigmatic cell; sp, septum; sy, synergid; tt, transmitting tract. Bars, 100 µm (A–E, G, H); 50 µm (F).

This activity was also observed in the pollen tubes (Fig. 4G), and it was especially high when the pollen tube burst occurred inside the embryo sac (Fig. 4H), staining almost all the embryo sac with a weaker GUS signal. This GUS expression in the embryo sac was never observed when p*AGP23*:GUS pistils were pollinated with wild-type pollen, only in embryo sacs fertilized with p*AGP23*:GUS pollen. This indicated that the GUS product present in the maternal embryo sac after fertilization was released by the burst of the pollen tube.

FISH confirms the GFP reporter lines patterns of expression

FISH was used to verify whether the GFP signals and GUS activity obtained with the p*AGP*:3GFP and p*AGP*:GUS fusions in fact reflected the real *AGP* gene expression. For this study, FISH was used to analyse two *AGP* genes: *AGP1* and *AGP12*. Hybridization signals for the *AGP1* antisense probe were detected throughout the septum, transmitting tract, and funiculus cells, as well as in the integuments surrounding the micropylar region of the embryo sac (Fig. 5A).

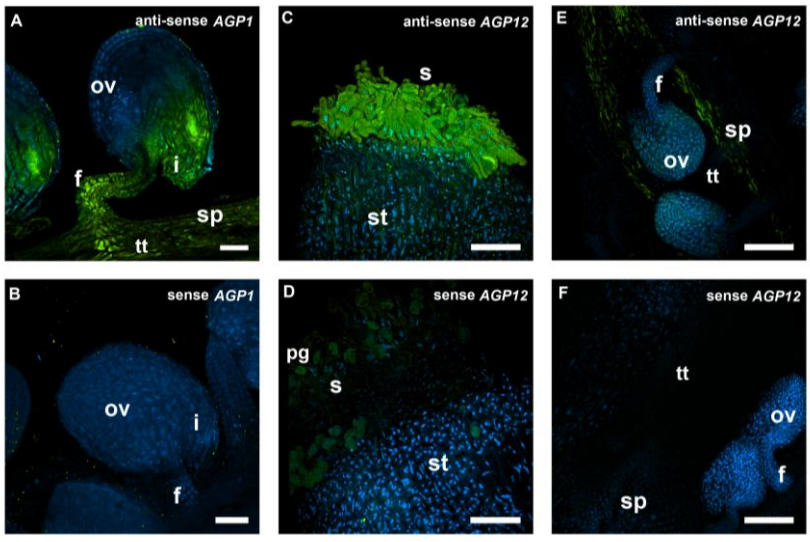

Figure 5 - FISH localization of *AGP1* and *AGP12* transcripts in Arabidopsis pistil tissues. Merged images of FISH signals (green) and DAPI staining of nuclei (blue) are shown. (A) *AGP1* transcripts were detected in the funiculus, transmitting tissue, and integuments. (C, E) *AGP12* transcripts were localized in the stigmatic cells and along the septum tissues. (B, D, F) FISH controls with the sense probe for *AGP1* in ovules (B) and for *AGP12* in stigma (D) and ovules (F). All

the flowers used in these observations were at stages 12 and 13 according to Smyth *et al.* (1990). f, Funiculus; i, integuments; pg, pollen grain; ov, ovule; s, stigma; sp, septum; st, style. Bars, 25 µm (A, B); 75 µm (C–F).

The same experiment using the *AGP1* sense probe revealed the absence of hybridization signal along all the reproductive tissues (Fig. 5B). With the *AGP12* antisense probe, strong hybridization signals were detected in the stigmatic cells (Fig. 5C) and a weaker signal was observed across the style and the septum (Fig. 5E). The corresponding *AGP12* sense probe did not show any hybridization signals along the reproductive tissues (Figs. 5D, F).

DISCUSSION

AGPs selection

Bioinformatics analyses recently allowed the identification of 64 potential AGPs in Arabidopsis (Showalter *et al.* 2010). The present work started by analysing 26 of these: those with more information available. However, it is important to keep in mind that for all individual AGPs almost no information is available at the structural level. Sequence comparisons revealed a high level of similarity between amino acid sequences of AGP15, an AG peptide, and AGP1, a classical AGP, as well as the inclusion of the three lysine-rich AGPs in the same branch as the classical AGPs. These results pinpointed the artificial nature of the classification currently in use to organize this family of proteins. The availability of more data regarding AGP expression patterns in different plant species and more information regarding their functions may allow the classification of these proteins based on their functions and localization, rather than on their amino acid sequences similarities. However, there are still some pairs of AGPs that share a high degree of similarity between their amino acid sequences, and, simultaneously, display a similar expression pattern in the reproductive tissues, suggesting that they might act redundantly, such as the AGP16/AGP20, AGP1/AGP15, AGP5/AGP10, and AGP6/AGP11 pairs. AGP6/AGP11 is a pair of redundant AGPs involved in Arabidopsis pollen grain and pollen tube growth and development (Coimbra *et al.* 2009). A total of 11 AGPs were picked for further analysis: AGP1, AGP4, AGP7, AGP9, AGP10, AGP12, AGP15, AGP16, AGP23, AGP25, and AGP26. This group was selected by an *in silico* search of AGP genes that could be transcribed preferentially in pistils rather than in the stamens or seedlings. This selection was based on analyses of microarray data available for pistil and stamen tissues obtained from Genevestigator, using the Anatomy tool provided by this service (Zimmermann *et al.* 2004) and the eFP Browser (Winter *et al.* 2007). Although *AGP18* fits perfectly into this category, it was not selected as it is already well described (Acosta-García and Vielle-Calzada, 2004; Demesa-Arévalo and Vielle-Calzada, 2013). *AGP23* was chosen as a control, since it is only transcribed during pollen development (Costa et al., 2013c; Nguema-Ona et al., 2013). Although microarray data from Genevestigator also predicted its expression in whole flowers and pistils, our real-time RT-PCR data confirmed that *AGP23* was detected only in pollen, being highly downregulated in pistils and seedlings.

The validation of this selection through real-time RT-PCR allowed us to limit the number of *AGPs* selected for further analysis to four: *AGP1*, *AGP9*, *AGP12*, and *AGP15*. *AGP9* and *AGP15* are upregulated in the pistil and downregulated in the seedlings, and were selected for this reason. *AGP1* was also selected, although its transcripts show a higher upregulation in seedlings than in emasculated pistils, because it is phylogenetically close to *AGP15*. *AGP12* was chosen as one of the most upregulated *AGPs* in the pistil.

Regarding *AGP* gene localization in the Arabidopsis chromosomes, it was clear that the *AGPs* were randomly distributed over the Arabidopsis genome. This was the case for *AGP16* and *AGP20*, located, respectively, on chromosomes 2 and 3, and also for *AGP6*, located on chromosome 5, and *AGP11*, on chromosome 3, two *AGPs* that have already been shown to act redundantly (Coimbra *et al.* 2009). This is probably due to duplications in the genome, since most of these genes are included in segments of the respective chromosomes that have been subject to large duplications events (Blanc *et al.* 2000). This is consistent with the prediction that genetic redundancy may occur as a consequence of gene duplication (Kafri *et al.* 2009). Only the pairs of the most similar *AGPs*, *AGP4*/*AGP7* and *AGP1*/*AGP15*, have their genes positioned in the same chromosome but in opposed regions. It is plausible that some of the *AGP* genes acquired a certain degree of specialization, and are now expressed in different tissues under different conditions.

AGPs expression in the reproductive tissues

The results obtained in this work confirmed the specific and differential pattern of expression of AGPs predicted previously by immunolocalization studies, where several monoclonal antibodies, recognizing distinctive AGP glycosidic epitopes revealed the presence of these proteins throughout diverse tissues in different developmental stages in Arabidopsis (Coimbra *et al.* 2007). These results not only confirmed and complemented this previous study but also improved the information already available about AGP distribution throughout the reproductive tissues by identifying specific AGPs present in these tissues. In the study by Coimbra *et al.* (2007), no antibody labelling was detected in the stigmatic cells, which, as was shown here, are rich at least in *AGP1* and *AGP12*. Also, in the same study, no antibody labelling was detected in the funiculus of the ovules, whereas, in this study, the presence of several AGPs, such as *AGP1*, *AGP12*, and *AGP15*, was revealed in this tissue. This work illustrates the usefulness of these techniques in contrast to the use of monoclonal antibodies to detect AGPs. As expected

from quantitative PCR data, *AGP23* was expressed only in pollen grains and pollen tubes. Although the microarray data available from Genevestigator indicated that *AGP23* should be present in pistils, this was not observed here. The analysis of transgenic Arabidopsis plants carrying the p*AG23*:GUS and the p*AGP23*:NLS:3GFP constructs revealed that both reporters were detected in pollen, proving that AGP23 is specific to the pollen vegetative cell. The prediction of potential of expression of *AGP23* in flowers and pistils was most probably due to the high levels of *AGP23* expression in pollen grains contained in the samples used for those studies. Concerning the pistil, the manipulation of these tissues is complicated if the flowers are not in the correct stage of development, as it is easy to get pollen contamination in the stigma, misleading to some false-positive expression. A summary of the different approaches used to localize these AGPs and their differential patterns of expression in the reproductive tissues is shown in Fig. 6.

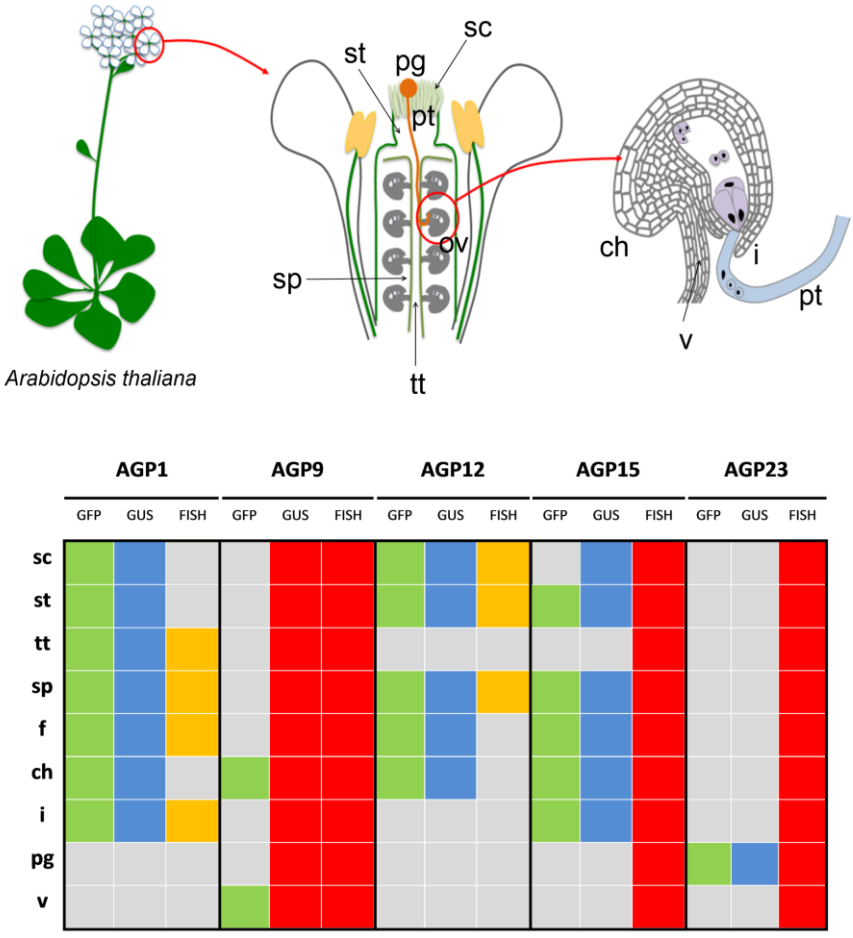

Fig 6 - A schematic representation of the reproductive structures and tissues of *A. thaliana* and the distribution of the five AGPs analysed in this study throughout the different tissues, regarding the different techniques used. GFP presence, green; GUS presence, blue; FISH positive, yellow; experiment not performed, red; absence of signal, grey. ch, Chalaza; f, funiculus; i, integuments; pg, pollen grain; sc, stigmatic cell; sp, septum; st, style; tt, transmitting tract; v, vasculature.

The FISH data obtained for *AGP1* and *AGP12* were partially consistent with the promoter analysis results shown for these two *AGPs*. The GFP expression driven by the *AGP1* and *AGP12* promoters revealed the presence of GFP signal in the chalazal tissues of the ovule, and, surprisingly, this was not observed in FISH results. This technique implies the analysis of whole-ovule amounts, making the tissue permeabilization more difficult in order for the probe to reach the most internal cell layers of the ovules, as is the case of the chalazal region (García-Aguilar *et al.* 2005; Hejátko *et al.* 2006). Still, we are aware that some regulatory elements of these two promoters might be missing, thus leading to the *AGP* misexpression in the chalazal tissues. Besides having regulatory sequences within the promoter itself, in eukaryotes there may be regulatory elements located tens of thousands of base pairs away from the start site, in introns, or even downstream from the coding sequence of the gene (Korkuć *et al.* 2014). Also, *AGP1* transcripts were not detected in the stigmatic cells or in the style by FISH analysis. It is important to underline the fact that the microarray data used and the FISH technique were performed with whole organs, while the promoter analysis referred to a spatial–temporal analysis, which was much more detailed. The older immunolocalization studies (Junqueira, 2007) did not detect the glycosidic AGP epitopes in the chalazal tissues. Although we are aware that the antibodies used identify only sugar epitopes from all AGPs, we may conclude, with some caution, that the accordance between the immunolocalization data and FISH results fortifies confidence in the use of antibodies to determine AGP localization.

AGP1 and *AGP12* expression in the stigmatic cells suggested the possible involvement of this protein in pollen–stigmatic cells interactions, and acquisition of pollen grain competence to initiate pollen tube growth. Losada and Herrero (2012) indicated a role for AGPs in supporting pollen tube germination, suggesting that the secretion of AGPs can be associated with the acquisition of stigma receptivity in apple flower. The same mechanism may occur with AGP12 and AGP1 in Arabidopsis. Also, in the early divergent angiosperm Trithuria, immunocytochemistry results suggest AGPs to be involved in attracting the pollen tubes through the stigmatic cuticle, as in most evolved angiosperms (Prychid *et al.* 2011), reinforcing our hypothesis.

The presence of *AGP1* and *AGP15* in the main female reproductive tissues through which the pollen tube grows until it reaches the embryo sac – the stigma, style, transmitting tract, septum, and funiculus – strengthens the putative role of AGPs in pollen tube growth and fertilization. Many early studies implied AGPs from the female tissues as

playing major roles in reproductive processes (Du *et al.* 1994; Cheung *et al*. 1995; Cheung and Wu, 1999; Wu *et al.* 2000; Coimbra *et al.* 2007). For example, TTS proteins (AGPs from *Nicotiana tabacum*) were shown to attract and promote pollen tube growth either *in vivo* or *in vitro*, nutritionally supporting its growth and providing it with guidance cues (Cheung *et al.* 1995; Wu *et al.* 2000). Wu *et al.* (1995) also revealed that the carbohydrate part of these TTS proteins forms an increasing gradient from the top to the bottom of the Nicotiana style, by the action of specific pollen tube hydrolases, which may have a chemotropic effect on growing pollen tubes. In Arabidopsis, the transmitting tract begins at the style between the stigma–style boundaries, extending to the base of the ovary (Crawford and Yanofsky, 2008). *AGP1* is mainly present along this transmitting tract, while *AGP15* is mostly present at the septum surrounding the transmitting tract. Since these two proteins are closely related to each other, this fortifies their possible redundant function in these tissues. *agp1* null mutants were analysed (data not shown) but revealed no visible phenotype. Most probably a double *agp1 agp15* mutant is needed to access their precise function. These AGPs might act in these tissues in a similar manner to the TTS proteins in Nicotiana. A study of the *NTT* gene in Arabidopsis has indirectly implied the involvement of AGPs in pollen tube guidance through the transmitting tract (Crawford *et al.* 2007). The *ntt* mutants lacked a functional transmitting tract and exhibited a reduced staining for acidic polysaccharides. Crawford *et al*. (2007) speculated that AGPs, acidic glycoproteins that are a main component of the transmitting tract, might be reduced in these mutants. It will be extremely interesting to check whether there is a control of *AGP* expression by this NTT zinc-finger transcription factor.

AGP1, *AGP9*, and more strongly *AGP12*, showed expression at the chalazal tissues of Arabidopsis ovules and at the cells located on the top of the vascular supply coming from the funiculus, as well as along this tissue. It is known that the main nutrient uptake into the endosperm occurs via the chalazal pole, with this being important for nutrient transfer from the maternal parent to the developing embryo (Debeaujon *et al.* 2003; Ingram, 2010). This may indicate the possible participation of these glycoproteins in nutrition or signalling between the vasculature and the embryo sac, endosperm, or embryo, being quickly mobilized. The incomplete correlation between GFP and GUS activity driven by the *AGP12* and *AGP1* promoters in this region and their transcript expression revealed the importance of analysing these AGPs at the protein level in future studies.

For double fertilization to take place, the pollen tube must travel a long and challenging pathway in order to reach its final destination: the micropylar entrance to the

embryo sac, where it will discharge, through one of the two synergids, two immotile sperm cells to fertilize the egg cell and the central cell, giving rise to the embryo and the endosperm, respectively, initiating a new generation (Márton and Dresselhaus, 2010). Along this narrow road, the pollen tube lengthens through a mucilage-rich extracellular matrix from the stigmatic cells, along the specialized transmitting tract cells, funiculus, and ovary integuments (Webb and Williams, 1988; Lennon *et al.* 1998). Although most of these studies showed that this extracellular matrix tract, through which the pollen tube travels, is rich in AGPs and pectins, to date only some specific molecules have been shown to function as pollen tube growth enhancers such as GABA in Arabidopsis (Palanivelu *et al.* 2003) and chemocyanin in *Lilium longiflorum* (Kim *et al.* 2003).

The results shown in this study support previous work where AGPs were proposed to be part of this pathway and to sustain pollen tube growth (Clarke *et al.* 1979; Herrero and Dickinson, 1979; Gell *et al.* 1986; Cheung *et al*. 1995). *AGP1*, *AGP12*, and *AGP15* (Fig. 6) are located along all these tissues and might well contribute to pollen tube growth from the top of the stigma to the base of the pistil, into the ovules, either by nutritionally supporting their growth, facilitating their movement, guiding them to their targets, or even by making them competent for pollen tube reception by the embryo sac. These hypotheses need further studies to fully assign AGP functions in these tissues, most probably involving studies with double or triple null mutants. It is interesting to note that we identified AGPs along the entire pollen tube pathway (stigma, style, and transmitting tract), showing that AGPs are most probably essential for all the different steps of pollen tube growth through the pistil. The molecular mechanism of action of AGPs and how they interact with other cell-wall and cell components is still elusive, although some enlightenment has recently been given to this matter (Costa *et al.* 2013a). One possibility may be related to the most recent finding that AGPs can act as calcium reservoirs, making calcium available temporarily in a developmental way (Lamport and Várnai, 2012). The importance of calcium in sexual plant reproduction is well known (Ge *et al.* 2007). One of the key characteristics of growing pollen tubes is a tip-focused calcium gradient maintained by the influx of extracellular calcium through calcium channels active at the extreme end of the growing tip (Feijó *et al.* 1995). AGPs may be regulating in some way the release of calcium along the pollen tube pathway, making calcium available for the pollen tubes to grow. Most likely, different AGPs play several different roles during different steps of the reproductive process, according to their localization and timing of expression (Fig. 6). Our results support and improve the study of these enigmatic and inscrutable glycoproteins in the sexual plant reproductive process, opening doors for new

pathways for the study of specific AGPs. Also, this type of analysis overcomes the main difficulty regarding the older immunolocalization AGP studies made by the use of monoclonal antibodies that detected only the glycosidic epitope of the AGPs, instead allowing the identification of a specific AGP in plant tissues.

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CHAPTER 4

"LOVE IS STRONG, AND YOURE SO SWEET": JAGGER IS ESSENTIAL FOR SYNERGID DEGENERATION AND POLYTUBEY BLOCK IN ARABIDOPSIS THALIANA

This chapter was based on:

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ABSTRACT

The key for successful double fertilization and the future development of a seed in flowering plants is the delivery of two sperm cells carried by a pollen tube into the embryo sac of an ovule. The embryo sac cells tightly control synergid cell death, and consequently the polyspermy block. Arabinogalactan proteins are highly glycosylated proteins that have long been implied in several steps of the reproductive process. We recently discovered that JAGGER, Arabinogalactan Protein 4, is essential to avoid the growth of multiple pollen tubes into one embryo sac in *Arabidopsis thaliana*. In *jagger*, a knock-out mutant for AGP4, the pistils show impaired pollen tube blockage as a consequence of the survival of the persistent synergid. JAGGER seems to be involved in the signalling pathway that leads to a blockage of pollen tube attraction. Our results present relevant data that will help to shed light into the mechanism responsible for preventing polyspermy in Arabidopsis and for safeguarding a successful fertilization of all ovules in one pistil, assuring the seed set and the next generation.

INTRODUCTION

In Angiosperms the successful development of a seed set is essential for the survival of any plant species and this depends on a tightly controlled delivery process of the two immotile sperm cells inside the embryo sac (ES) of the ovules. This process is carried out by the pollen tube (PT), which grows initially as a protuberance of the pollen grain in the stigma, and continues its crusade through the pistil tissues by a tip growing process, until it reaches the embryo sac (ES). Once nearby the ES, the PT is attracted by specific molecules, such as the AtLures (Takeuchi and Higashiyama, 2012), produced by the synergid cells. After entering the ES though the synergid filiform apparatus (Drews and Koltunow 2011; Kessler and Grossniklaus 2011) or nearby this structure, as suggested by a recent study (Leshem *et al.* 2013), the PT arrests its growth and bursts inside one of the synergids releasing the sperm cells (Dresselhaus and Franklin-Tong, 2013). Afterwards, the disrupted synergid starts to degenerate and each one of the sperm cells fuses with the central and the egg cell, giving rise to the endosperm and the embryo, respectively (Russell, 1992).

Little is known about the mechanisms that regulate the attraction and/or repulsion of PTs into the ovules (Dresselhaus and Sprunck, 2012). Typically, a PT delivers a pair of sperm cells that fuse with the egg and central cell, effecting double fertilization. But, if one of the sperm cells is unable to fuse with one of the female gamete cells, or even both, the ES has a back-up mechanism to avoid double fertilization failure and assure a successful seed set formation. It is known that if one of the fusions fails, the ES continues to attract more PTs until one is finally capable of delivering enough functional sperm cells and achieve double fertilization (Beale *et al.* 2012; Kasahara *et al.* 2012). This is attained by the maintenance of one functional synergid, by avoiding its death. Recent studies showed that after successful double fertilization, each one of the fertilized female gametes, independently, trigger a signalling cascade that leads to the programmed cell death (PCD) of the persistent synergid consequently blocking the production of more molecules for PT attraction (Beale *et al.* 2012; Maruyama *et al.* 2013). Simultaneously, Völz *et al.* (2013) discovered that ethylene is involved in the signal transduction cascade that results in PCD of the persistent synergid. *ein3* and *ein2* mutants, defective in the ethylene signalling pathways, revealed their importance for synergid cell death and the establishment of a PT block (Völz *et al.* 2013). How this two mechanisms may interact or not, it is yet not understood (Schnittger, 2013). This step is crucial to avoid the attraction of multiple PTs

into one embryo sac, a phenomenon designated by polytubey (Beale *et al.* 2012). The female gametophyte controls the growth of the PT as well as the timing and place for sperm cell discharge and fertilization occurrence (Kessler and Grossniklaus, 2011).

Despite these recent studies, various mutants with defects in PT attraction had been identified before. In *feronia* and its alelle *sirène*, PTs enter the ES but fail to rupture and deliver the sperm cells, overgrowing inside the ES (Huck *et al.* 2003; Rotman *et al.* 2003). These mutants are also able to attract supernumerary PTs (Escobar-Restrepo *et al.* 2007). *FER* encodes a receptor-like kinase belonging to the *Cr*RLK1L-1 subfamily of kinases. It is predicted to act as a receptor that somehow senses the PT arrival, activating a signalling cascade that will mediate synergid-PT interactions after PT entrance (Escobar-Restrepo *et al.* 2007). Another protein involved in PT reception is LORELEI, a glycosylphosphatidylinositol (GPI) – anchored protein (Capron *et al.* 2008; Tsukamoto *et al.* 2010). *lorelei* presents a phenotype similar to that of *feronia*/*sirène*, where the PT enters the ES but does not release the sperm cells, avoiding fertilization and attracting supernumerary PTs. LORELEI is thought to be involved in the activation of the FER/SRN signalling cascade (Kessler and Grossniklaus, 2011). In every known mutant until now, where the fertilization of both female gametes is not accomplished, the ES continues to attract more PTs until it assures a successful double fertilization. Only in the mutants defective in the ethylene-signalling pathway, double fertilization occurs normally and the ES continues to attract supernumerary PT's.

Arabinogalactan proteins (AGPs) constitute a class of highly glycosylated proteins, ubiquitous in plants; most of them are predicted to be tethered to the plasma membrane by a GPI-anchor (Seifert and Roberts, 2007). For several years they were directly or indirectly implied in reproductive process of several species (Pereira *et al.* 2015). Here we describe the *jagger* mutant phenotype, an *AGP4* mutant, named after the rock'n'roll god Mick Jagger, who "can't get no satisfaction", because it attracts more than one PT per embryo sac in its ovules. This mutant has a sporophytic defect, since the ES, the pollen grain, PT and sperm cells develop normally. Also, double fertilization is successfully achieved when the first PT bursts inside the ES, pointing to some failure in the communication between the fertilized egg and/or central cell and the persistent synergid involving the sporophytic tissues. The persistent synergid does not undergo programmed cell death after double fertilization, as expected, and consequently continues to attract more PTs into the ES. We hypothesize that JAGGER either via its carbohydrate moiety or the whole protein, acts as an intermediary in the signalling pathway that triggers the persistent synergid cell death.

MATERIAL & METHODS

Plant material and growth conditions

Arabidopsis thaliana wild-type ecotypes *Col-0* and *No-0*, along with two *agp4*/*jagger* mutant lines were used in this work, *jagger1* is the RIKEN pst20518 (Ito *et al.* 2005), *jagger2* is the GABI-Kat 34A10 line (Kleinboelting *et al*. 2012. The marker lines for the central cell (*DD7*:GFP), egg cell (*DD45*:GFP) and synergid cell (*MYB98*:GFP and *DD2*:GFP) were kindly shared by G. Drews (Steffen *et al.* 2007). The marker line for the pollen tube and the sperm cells, p*LAT52*:GFP-*H3.3*:mRFP was kindly shared with us by S. Sprunck.

The plants were sown directly on soil and grown in an indoor growth facility with 60% relative humidity and a day-night cycle of 16 hours light at 21°C and 8 hours darkness at 18°C. For crosses with dehiscent anthers closed flower buds were emasculated 24-48 hours before pollination, at stage 11-12, according to Smyth *et al.* (1990). Gametophytic cell marker lines bearing the *jagger* mutant allele were obtained by crossing *jagger* with the *DD7*:GFP, *DD45*:GFP and *MYB98*:GFP lines, and maintained by self-crossing. For PAT (Phosphinotricin-Acetyltransferase) selection the seedlings were sprayed with 200 mg l-1 glufosinate ammonium (BASTA®; Bayer Crop Science) supplemented with 0.1% Tween 20 for three or four times, every two days, during a ten day period.

Genotyping

For the jagger1 mutant line a PCR-based approach was employed to confirm the *Ds* insertion and to identify the homozygous mutant. Genotyping primers LP-GK-134A10, RP-GK-134A10 and DS34 were used (Supplemental Table 1). For the jagger2 mutant line the same approach was used. Genotyping primers were LP-GK-134A10, RP-GK-134A10 and 08409. All primers sequences are listed in Supplemental Table 1.

Constructs generation and plant transformation

A DNA fragment encoding the promoter region of *JAGGER* was amplified by PCR

using the primers AtP_4390 and AtP_4391 (Supplemental Table 1), including 3051 bp upstream of the 5´UTR. The amplified fragment was cloned into pDONR207™ (Invitrogen). The promoter fragment was transferred into the binary vector pBGWFS7 (Karimi *et al.* 2002) in order to obtain the *JAGGER*$_{pro}$:GUS construct. An overexpression vector was obtained using a 763 bp DNA fragment corresponding to the *JAGGER* coding sequence. This DNA fragment was amplified by PCR using the primers AtP_4486 and AtP_4487 (Supplemental Table 1). The amplified fragment was cloned into pDONR207™ (Invitrogen) and thereafter transferred into the overexpression vector pB2GW7 (Karimi *et al.* 2002) to obtain the 35s$_{pro}$:*JAGGER* construct. All constructs were confirmed by DNA sequencing. *Arabidopsis thaliana* Col 0 were transformed by the floral dip method (Clough and Bent, 1998).

Pollen tube staining with Aniline Blue

Arabidopsis flowers were fixed in 10% acetic acid in ethanol for approximately 16 h, washed 3 times with water and softened with 1 M NaOH for another 16 h, washed 3 times with water, and left overnigth with 0.1% decolorized aniline blue at 4ºC. The specimens were observed with a Zeiss Axiophot D1 microscope. Images were captured on an Axiocam MRc5 camera (Zeiss) using the Axiovision program (version 4.1).

Preparation of live plant material for microscopy

Emasculated pistils were observed 2 days after pollination, were kept in 50 mM sodium phosphate buffer (pH 7.5) and dissected under a stereomicroscope (Model C-DSD230, Nikon) using hypodermic needles (0.4 x 20 mm; Braun). The opened carpels and the ovules that remained attached to the septum were maintained in mounting medium and covered with a cover slip. Pistils pollinated with the pLAT52:GFP-H3.3:mRFP pollen were observed under an inverted Nikon microscope.

For phenotypic characterization, ovules at different developmental stages were cleared and analysed as described previously (Brambilla *et al.* 2007). Samples were observed using a Zeiss Axiophot D1 microscope equipped with differential interference contrast optics. Images were captured on an Axiocam MRc5 camera (Zeiss) using the Axiovision program (version 4.1).

Preparation of fixed plant material for light microscopy

Pistils were fixed in 2 % paraformaldehyde and 2·5 % glutaraldehyde in phosphate buffer (0.025 M, pH 7, with one micro drop of Tween 80), placed under vacuum for 1 h and then left at 4 °C overnight. After dehydration in a graded ethanol series, the material was embedded in LR White embedding resin (London Resin Company Ltd, London, UK). Thick sections (0.5 µm) were obtained with a Leica Reichert Supernova microtome placed on glass slides, and stained with a solution of 0.05% toluidine blue in a phosphate-citrate buffer (pH 4 - 6) and double stained with 1% aqueous safranin O for observation under light microscopy. Bright-field observations were made on a Leica DMLB epifluorescence microscope (objectives were Leica N-Plan). Images were captured with a ProgRes® MF cool (Jenoptik, Jena, Germany) in automatic exposure mode, and processed with ProgRes® CapturePro 2.8.8 software.

GUS assays

GUS assays were performed on inflorescences as described in Liljegren *et al.* (2000), overnight. After chemical GUS detection, the samples were incubated in clearing solution [160g of chloral hydrate (Sigma-Aldrich), 100mL of water, and 50mL of glycerol] and incubated at 4ºC ON. A Zeiss AxioImager AZ microscope equipped with differential interference contrast (DIC) optics was used. Images were captured with a Zeiss Axiocam MRc3 camera using Zen Imaging Software.

RNA extraction, cDNA synthesis and Real Time RT-PCR

Total RNA from emasculated pistils extracted using PureZolTM RNA Isolation Reagent (Bio-Rad) following the manufacter's instructions. DNA was removed by a DNase (Thermo Scientific) treatment. The isolated RNA samples were reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and oligo$(dT)_{18}$ primers to initiate the reactions, following the manufacter's intructions. cDNA was amplified using the SSoFast™ SYBR® Green Supermix on an iQ5™ Real-Time PCR Detection System (Bio-Rad) using the primers listed in Supplemental Table 1. Real-time RT-PCRs were run in triplicates. After 3 min at 95°C followed by a 10 s denaturation step at 95°C, samples were run for 40 cycles of 10 s at 95°C and 30 s at 60°C. After each run, a dissociation curve was acquired to check for amplification specificity by heating the samples from 60 to

95°C. Serial dilutions of pure genomic DNA from Arabidopsis ecotype Columbia were used to set up a calibration curve, which was used to quantify plant DNA in each sample. At the end of the PCR cycles, data were analysed with iQ5 2.0, Standard Edition Optical System Software v2.0.148.060623 (Bio-Rad).

In situ hybridization

For in situ hybridization analysis, *A. thaliana* flowers were fixed and embedded in paraffin as described previously. Sections of plant tissue were probed with digoxigenin-labeled *JAGGER* antisense RNA corresponding to nucleotides 35 to 399 (See Supplemental Table 1 for primers used for probe synthesis). Hybridization and immunological detection were performed as described previously (Masiero *et al.*, 2004).

Yeast Two Hybrid

The predicted peptide core of JAGGER, followed by a stop codon, was individually cloned into the pGBTKT7 vector (Clontech, Palo Alto, CA), modified at the XmaI site to include a Gateway cassette (primers used are described in Supplemental Table 1). The bait construct was introduced in the α-Y187 strain (Gietz *et al.* 1992). YPAD, SD, and appropriate dropout media have been described previously (Sherman, 2002). The bait strain was mated with a normalized *Arabidopsis thaliana* total plant cDNA library cloned in pGADrec (Sommer and Masiero, unpublished data) and introduced in the α-AH109 yeast strain (Clontech). Colonies that grew on all selective media (−Trp-Leu-Adenine-His and supplemented with 15 mM 3-Amino-1,2,4-triazole) were further characterized. The pGADrec plasmids were rescued and cDNA insert was amplified by PCR and sequenced.

Image processing

All images were processed for publication using ImajeJ (Schneider *et al.* 2012) and Paint.NET (Copyright© dotPDN LLC and Rick Brewster).

Statistical analysis

Data were statistically treated using the graphpad software (www.graphpad.com).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AGP4 (At5G104380), RUB1 (At4G36800) and ACT8 (At1G49240). All the the accession numbers for genes discussed, resulting from Y2H experiments, are listed on the supllementary table 2.

RESULTS

Isolation and characterization of the jagger1−/− and jagger2-/- homozygous mutant lines

A DS transposon insertion line from RIKEN library was selected (pst20518) as well as a transfer DNA (T-DNA) insertion line from the GABI-Kat library (GK-134A10). pst20518 contained the DS transposon insert in the JAGGER coding sequence, while GK-134A10 contained a T-DNA insertion in the JAGGER 3´UTR (Supplemental Fig. 1A) as confirmed by PCR and sequencing. Both homozygous lines were isolated, *jagger* -/- and *jagger2*-/-. Copies of the inserts were amplified with the gDNA (Supplemental Figs. 1B, C). JAGGER transcript was not detectable by quantitative reverse transcription (qRT)-PCR in jagger1-/-, but in *jagger2*-/- a residual level of transcript was detected (Fig. 1) revealing this line to be a knockdown mutant rather than a knockout mutant like *jagger1*-/-.

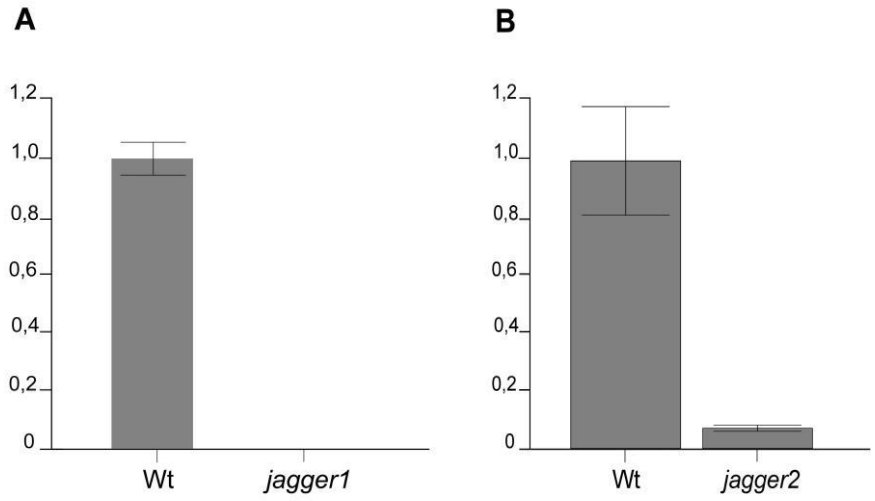

Figure 1 – Relative expression of *JAGGER* in wild-type flowers, *jagger1*-/- and *jagger2*-/- mutant flowers.
A. *jagger1*-/- revealed no *JAGGER* expression in flower tissues. **B.** *jagger2*-/- revealed a residual expression of *JAGGER* in flower tissues. The relative gene expression was measured using stably expressed reference genes (At4g27960 and At1g06780) in three biological samples with similar results. The data correspond to the ratio of the expression in wild-type or jagger-/- compared to the wild-type and are the mean ± sd of three technical replicates of a biological sample.

The vegetative organs and the adult plant of *jagger1*−/− and *jagger2*−/− did not

show any visible phenotype or growth defect compared with wild-type plants. To determine whether the male or the female gametophytes were affected in the mutant, reciprocal crosses were conducted between wild-type and the heterozygotes mutants. As shown in Table 1, genetic analyses pinpoint that *jagger* male and female gametophytes transmission efficiencies are not affected.

A - *jagger1* transmission efficiency

Transmission	Cross	+/+	+/*jag1*	Expected
Male	+/+ X +/*jag1*	55	65	60
Female	+/*jag1* X +/+	63	52	57,5

The Chi-square statistic is 1.8813. The P value is 0.170188. This result is not significant at $p < 0.05$.

B – *jagger2* transmission efficiency

Transmission	Cross	+/+	+/*jag2*	Expected
Male	+/+ X +/*jag2*	48	72	60
Female	+/*jag2* X +/+	61	58	65

The Chi-square statistic is 3.054. The P value is 0.080538. This result is not significant at $p < 0.05$.

Table 1 - T-DNA transmission analysis of *jagger1* (A) and *jagger2* (B).

A. The number of *jagger1* (+/*jag1*) and wild-type (+/+) plants among test cross progeny and the percentage of *jagger1* mutant progeny (% *jag1*/+). The transmission efficiency (TE) represents the percentage of *jagger1* mutant alleles successfully transmitted through male or female gametes. Chi-square test for a 1:1 segregation hypothesis was calculated. **B.** The number of *jagger2* (+/*jag2*) and wild-type (+/+) plants among test cross progeny and the percentage of *jagger2* mutant progeny (% *jag2*/+). The transmission efficiency (TE) represents the percentage of *jagger2* mutant alleles successfully transmitted through male or female gametes. Chi-square test for a 1:1 segregation hypothesis was calculated.

JAGGER expression pattern in reproductive tissues

The *JAGGER* promoter fused to GUS was used in order to allow the detection of the expression pattern of this gene in the female reproductive tissues. All the flowers analysed were between stages 12 and 13 according to Smyth *et al.* (1990). GUS activity driven by the *JAGGER* promoter was strong in the transmitting tract cells (Figs. 2A-C), the stigmatic cells (Fig. 2B), and also in the ovules integuments near the micropyle (Figs. 2D-G) in mature ovules. Inside the embryo sac GUS activity was detected in the region corresponding to the synergids and egg cell localization, the so-called egg-apparatus (Fig.

2D-G). No GUS activity was detected in any other reproductive tissues. This GUS activity disappeared right after fertilization and beginning of seed development (Fig. 2E).

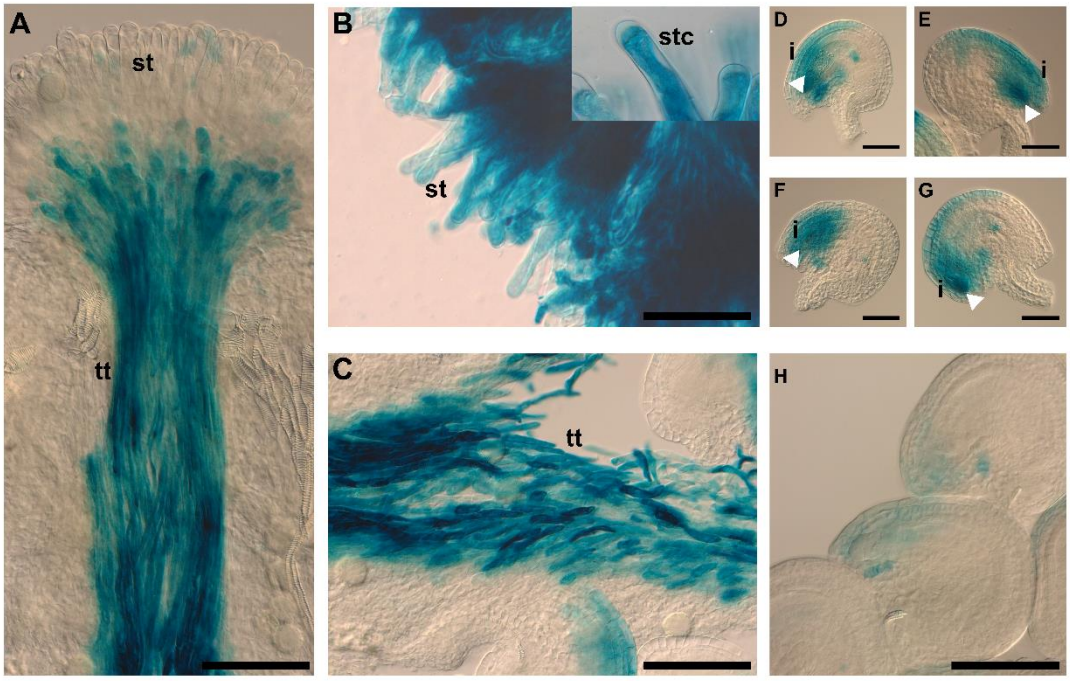

Figure 2 – Histochemical localization of GUS activity in transgenic Arabidopsis pistil tissues expressing the *JAGGER*$_{pro}$:GUS fusion gene.

A. GUS activity driven by the *JAGGER* promoter is detected in the stigmatic cells (st) and in the transmitting tract (tt) cells. **B.** Detail of GUS activity detected in the stylar and stigma cells. **C.** Strong GUS activity detected in the transmitting tract cells in more detail. **D – G.** GUS activity driven by *JAGGER* in the integuments near the micropyle and inside the embryo sac in the egg apparatus (white arrowhead). H. Developing seeds after fertilization where GUS activity is reduced comparing to the other stages of ovule development. Flowers of stage 12 and stage 13 (Smyth et al., 1990) were used in this study. I – integuments; st – stigma; tt – transmitting tract. Bars: 100 µm in A – C, H; 20 µm in D – G.

In situ hybridization was performed in order to confirm if the GUS activity pattern obtained with JAGGER$_{pro}$:GUS reflected the real JAGGER expression. As it can be seen in Figure 3 JAGGER transcripts were detected in the same tissues where the GUS activity was detected. Hybridization signals for the JAGGER anti-sense probe were detected throughout the stigma, style, and ovule integuments as well as inside the embryo sac (Fig. 3 A-C). The same experiment with the anti-sense probe did not show any hybridization signals along the reproductive tissues of *jagger* samples (Fig. 3 D, E). All the flowers used in these observations were at stages 12 and 13 according to Smyth *et al.* (1990).

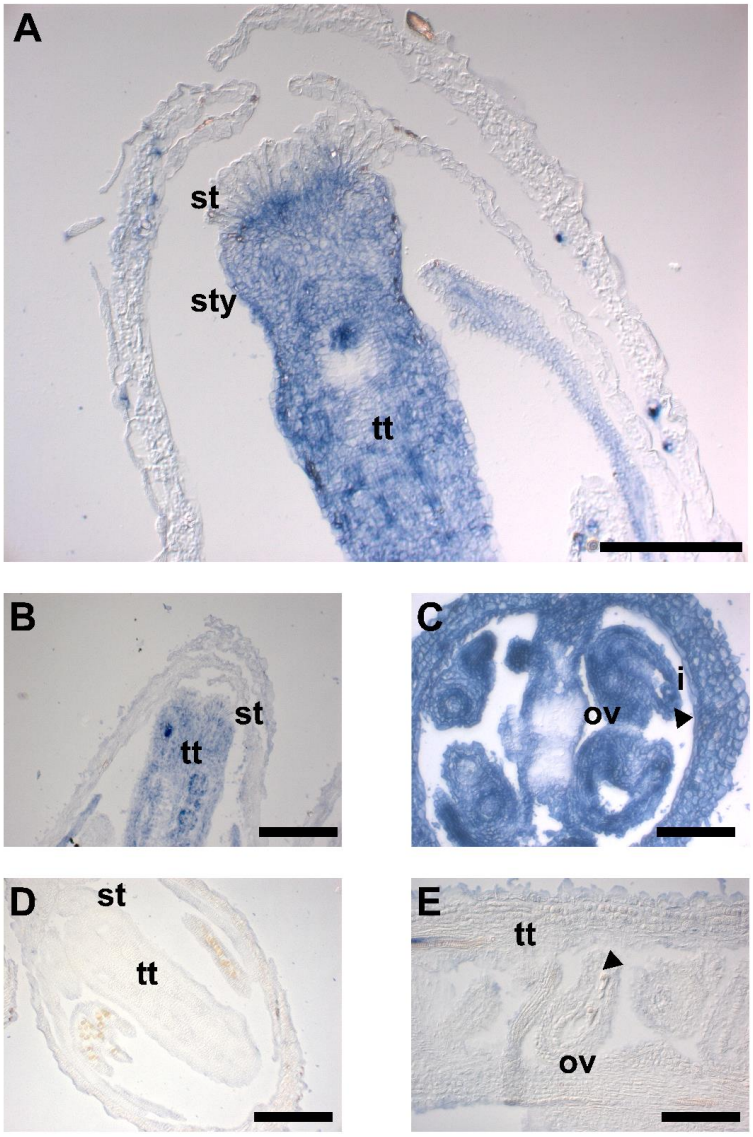

Figure 3 – *JAGGER* expression in wild-type and *jagger* pistils' sections detected by *in situ* hybridization.

A. Longitudinal section of a wild-type pistil revealing *JAGGER* expression in the transmitting tract cells in the style and the rest of the pistil. **B.** A closer detail showing *JAGGER* expression in wild-type stigma and transmitting tract cells. **C.** Wild-type pistil transversal section showing *JAGGER* expression in the ovules integuments and in the egg apparatus. **D, E.** *jagger* pistil sections revealing the absence of *JAGGER* expression in the mutant pistils. Flowers of stage 12 and stage 13 (Smyth *et al.* 1990) were used in this study. I – integuments; ov – ovule; st – stigma; sty – style; tt – transmitting tract. Bars: 100 µm in A, B, D; 50 µm in C, E.

In vivo pollen tube growth

Hand-pollination of emasculated wild-type pistils with *jagger*-/- pollen grains, followed by aniline blue staining, showed that the *jagger*-/- PTs successfully grow through the transmitting tract cells and reached the embryo sac of the *jagger*-/- ovules

accomplishing double fertilization, completing their journeys by reaching the bottom of the ovary 24 h after pollination. In the reciprocal crosses wild-type PTs also grow normally along the transmitting tract cells reaching the embryo sac micropyle, entering and releasing the sperm cell to accomplish double fertilization. In the *jagger* mutant pistils, it was usual to observe more than one PT entering the same embryo sac (Figs. 4 A, B), unlike the wild-type flowers, where each embryo sac received only one PT (Fig. 4C).

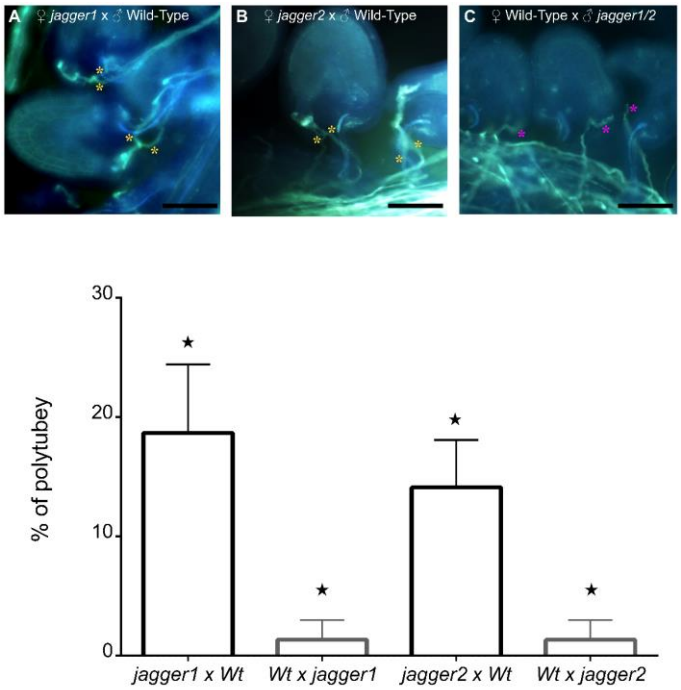

Figure 4 – Aniline blue staining of reciprocal crosses between *jagger1*/*jagger2* and wild-type flowers and percentage of polytubey observed.

A. Cross between a female *jagger1-/-* flower and wild-type pollen where two polytubey situations can be observed as pointed out by the yellow asterisks. **B.** Cross between a female *jagger2-/-* flower and wild-type pollen where two polytubey situations can be observed as pointed out by the yellow asterisks. **C.** Cross between a female wild-type flower and *jagger1/2* pollen where normal single PTs enter each ovule as pointed out by the pink asterisks. **D.** Percentage of polytubey observed in reciprocal crosses between *jagger1-/-* pistils and wild-type pollen grains (*jagger1* x Wt) and *jagger2-/-* pistils and wild-type pollen grains (*jagger2* x Wt). Wt – Wild-type. Bars represent the standard deviation for each condition. Asterisks indicate statistically significant differences between the reciprocal crosses determined by the Student's t-test ($P<0.0001$; n=600 ovules in *jagger1* x Wt; n=900 in Wt x *jagger1*, *jagger2* x Wt and Wt x *jagger2*). Bars: 50 µm in A, B and C.

In the *jagger-/-* it was always observed no more than two PTs entering the same embryo sac. This phenomenon is called polytubey (Beale *et al.* 2012). To better analyse this phenotype the number of polytubey observed in wild-type and *jagger* pistils was calculated and statistically considered. It was observed 18,7% ± 5,7 of polytubey in *jagger1-/-* pistils pollinated with wild-type pollen and 1,3% ± 1,6 in wild-type pistils pollinated with jagger1-/- pollen grains ($P<0,0001$; n=600 ovules for jagger1-/- x Wt and

n=900 ovules for Wt x jagger1-/-). For the second allele 14,1% ± 3,9 of polytubey was observed in crosses between *jagger2-/-* pistils and wild-type pollen, while in the reciprocal cross 1,3% ± 1,6 was the percentage of polytubey observed (P<0,0001; n=900 ovules for both crosses), as depicted in Figure 4D. The observation of the same phenotype in both alleles strongly suggests that JAGGER is responsible for this defect in PT attraction.

Seed set and fertilization analysis

For *jagger1* and 2 the number of viable seeds, aborted ovules and seeds inside the siliques were analysed, revealing no significant difference with the wild-type siliques grown in the same conditions. To check if gamete fusion was occurring normally in *jagger* a marker line kindly shared with us by S. Sprunck was used containing the construct LAT52$_{pro}$:GFP-H3.3mRFP.

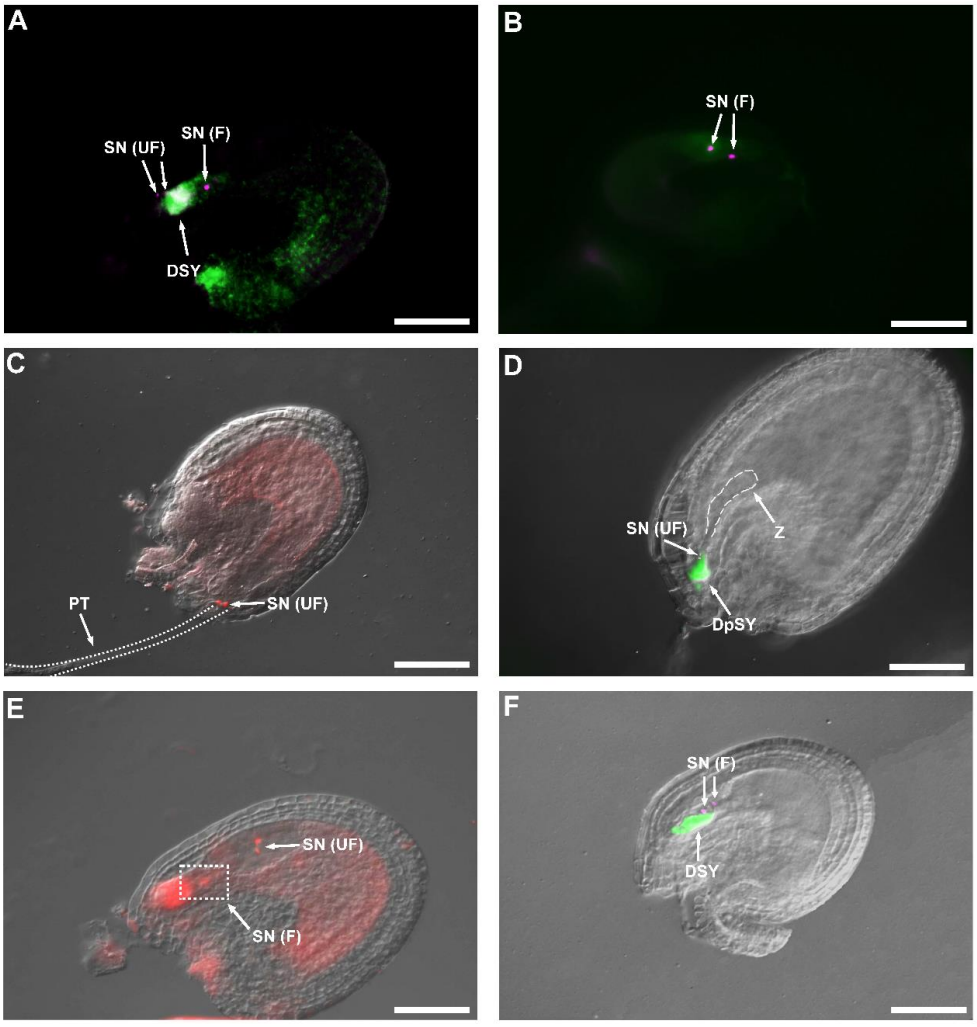

Figure 5 – Crosses between *jagger-/-* pistils and pollen grains containing the construct LAT52pro:GFP-H3.3mRFP reveal that fertilization occurs normally but PT block is impaired.

A. *jagger-/-* ovule receiving two pairs of sperm cells. The green signal is released from the burst of the PT causing degeneration of the synergid (DSY); the first sperm nuclei (SN, magenta signal) fused (F) with the egg cell and central cell nuclei, only one fusion is visible here. The second pair of SN does not separate and persist unfused (UF). **B.** *jagger-/-* embryo sac receiving one PT with a pair of SN normally released and fused with the central and egg cell**. C – F** Differential interference contrast (DIC) and fluorescence images are overlaid. **C.** *jagger-/-* developing seed receiving a second pollen tube (PT) with a pair of SN unfused at the embryo sac entrance. The PT is in a different plane and the dashed lines represent the PT entering the embryo sac. **D.** *jagger-/-* developing seed with a visible zygote (Z, highlighted by the dashed lines) receiving a second pair of SN unfused, the green signal represents the burst of the PT inside the persisting synergid leading to its degeneration (DpSY). **E.** *jagger-/-* developing seed that has already received a pair of SN that fuses normally (highlighted by dashed square) and a second pair of SN unfused in the region corresponding to the developing endosperm. **F.** Wild-Type ovule receiving a pair of SN represented by the magenta colour and fused normally with the central and egg cell; the green signal represents the PT burst inside the receptive synergid and the beginning of its degeneration (DSY). Bars: 20 µm.

The plants bearing this construct produced pollen grains expressing GFP through all the pollen grain and the PT, and expressed mRFP in the sperm cells. Reciprocal crosses were made between *jagger1* and 2 pistils with $LAT52_{pro}$:GFP-H3.3mRFP pollen. The reciprocal crosses revealed no differences between the sperm cells fusions with the egg and the central cell in wild-type or *jagger* plants (Fig. 5B, F). Extra pairs of sperm cells were observed arriving at the micropyle region (Fig. C,D), inside the egg apparatus (Fig. 5A) and entering the already developing endosperm (Fig. 5E). This reinforces the observations of multiple PTs entering one embryo sac.

Embryo sac cells develop normally in jagger-/-

To check if the female gametophytic cells involved in reproduction were developing normally, *jagger-/-* plants were crossed with independent transgenic plants containing the following constructs: *MYB98*:GFP, *At2g20595*:GFP/ dd7:GFP and *EC1.2*:GFP/ dd45:GFP. These lines were kindly shared with us by G. Drews. After crossing each one of the lines with *jagger-/-* and obtaining homozygous plants for both, marker line and T-DNA insertion mutants, no differences were observed between the control plants expressing the GFP reporter under the respective promoter and the ones crossed with *jagger-/-* (Supplemental Fig. 2). *MYB98*:GFP plants showed GFP expression in all the synergids as well as the jagger-/- plants crossed with this marker lines (Supplemental Figs. 2A, B). dd7:GFP plants showed GFP expression in the central cell, and the same pattern of expression was observed in the cross with jagger-/- plants (Supplemental Figs. 2C, D). GFP driven by the

DD45 promoter lead to its expression in the egg cell, and the same expression pattern was observed in crosses with *jagger*-/- (Supplemental Figs. 2E, F). Besides these experiments, *jagger*-/- and wild-type flowers were embedded in LR-white resin and cross sections of these inclusions were obtained. The observation of these sections under the optical microscope revealed no differences between the *jagger*-/- and the wild-type ovules development. As it can be seen in Figure 6A and 6B the synergids, the egg cell and the central cell seem to develop normally just as the -ype ovules shown in Figure 6D and 6E. Moreover, in Figure 6C it is possible to observe the entrance of a PT into one of the embryo sac synergids, as well as one of the sperm cells carried inside it.

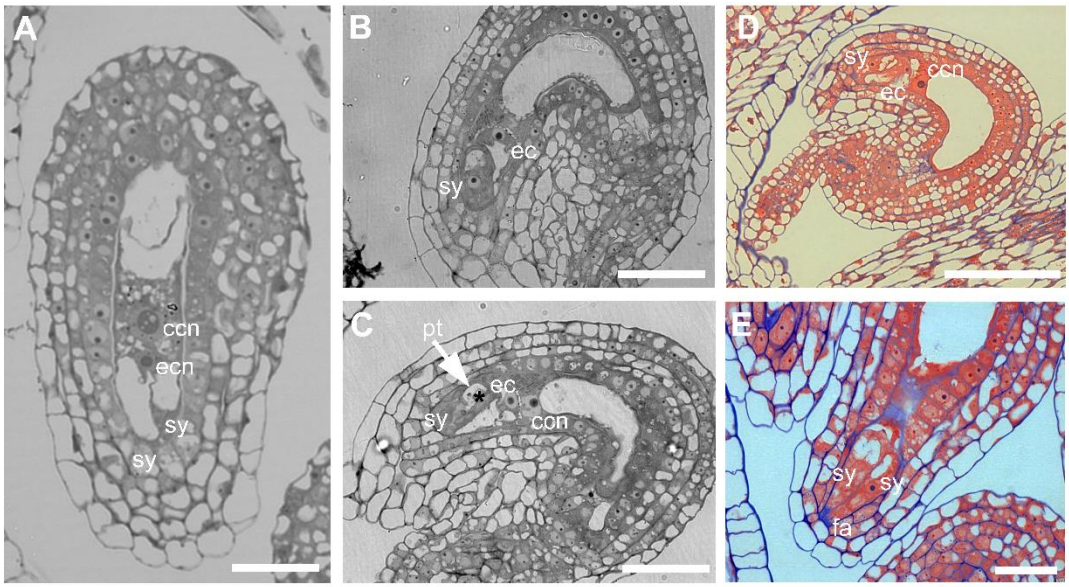

Figure 6 – Cross sections of LR-White embedded ovules of *jagger* and Wt plants.

A. Section of a *jagger* ovule showing normal development of the female gametophytic cells central cell, egg cell and synergids. **B.** Section of a *jagger* ovule showing normal synergid and egg cell development. **C.** Section of a *jagger* ovule showing a PT entering the embryo sac (white arrow) and one of the sperm cell nuclei (marked with an asterisk). Normal synergid, egg and central cell nucleus are visible. **D.** Section of a wild-type ovule showing normal development, with the central cell, the egg cell and one of the two synergids in their common positions. **E.** Section of of a wild-type ovule showing in more detail the normal development of the two synergids and their filiform apparatus. Flowers of stage 12 and stage 13 (Smyth *et al.* 1990) were used in this study. Ccn – central cell nucleus, ec – egg cell, ecn – egg cell nucleus, fa – filiform apparatus, sy – synergid, pt – pollen tube. Bars: 30 µm.

The jagger mutation affects synergid cell death

Transgenic plants carrying the $35s_{pro}$:*JAGGER* construct were analysed, only three lines survived to the BASTA treatment, 2 independent lines corresponded to plants overexpressing the JAGGER gene, lines 1 and 2 (Supplemental Fig. 3). These lines revealed a normal development and pollen grains developed normal PTs when germinated on the stigma (Fig. 7A). When aniline blue stained pistils were checked for PT growth a considerable amount of ovules were unfertilized and presented callose deposition inside the embryo sacs, near the micropyle region, corresponding to the synergids localisation (Fig. 7C, D), and only some few ovules revealed a normal PT growth inside the embryo sac (Fig. 7B). Not only these cells, but also the nucellar cells and integumentary cell near the micropyle presented the same kind of staining, indicating a premature cell death (Fig. 7C, D). When the seed set was checked in siliques fixed with ethanol, large areas with no seeds most probably corresponding to aborted ovules and seeds were detected (Fig. 7E).

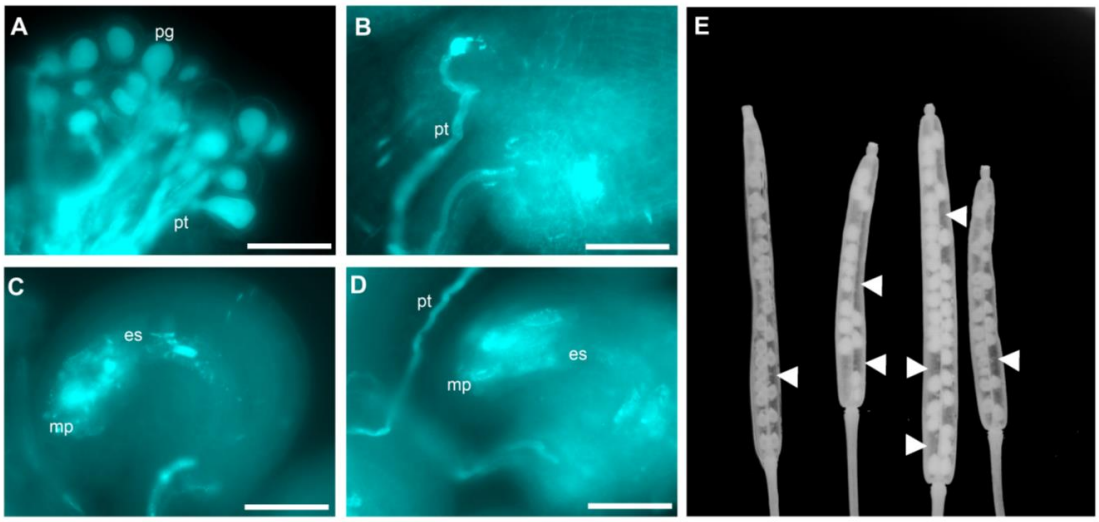

Figure 7 – Aniline blue staining of $35s_{pro}$:*JAGGER* overexpressing plant female organs and fixed siliques from the same plants.

A. Pollen grains germinating correctly on the top of the stigma of a pistil from a plant overexpressing JAGGER. PTs grow normally trough the style tissues. Only the callose stained by the aniline blue is visible. **B.** PT entering the embryo sac of an ovule. **C.** Callose accumulation inside the embryo sac and the micropyle region of the ovule, including the adjacent integuments. **D.** Callose accumulation inside the embryo sac and the micropyle region of an ovule with a PT passing nearby, but not entering the ovule. C. Fixed siliques showing a reduced number of seeds per silique (white arrowheads). Es – embryo sac, mp – micropyle, pg – pollen grain, pt – pollen tube. Bars: 50 µm.

Given the phenotype of these plants overexpressing *JAGGER*, we decided to check

if the cell death of the persisting synergid was following normally. If the persisting synergid was still alive, it would still be producing PT attractants, even after normal fertilization of the female gamete cells, and it could explain the polytubey phenotype. Analysis of *jagger2-/-* cleared ovules 24 hours after pollination with wild-type pollen revealed that the persisting synergid was still alive even after successful fertilisation and beginning of zygote formation. It is clear the presence of an extra nucleus in the micropylar region of the ovules, near the zygote (Fig. 8A - C). In Figure 8C it is visible a persisting synergid in an eight-nuclear endosperm, where only six of these nuclei are visible in the same plane. In wild-type ovules, analyzed at the same stage, and pollinated with *jagger2-/-* no surviving synergid nuclei could be detected in the micropylar region of the ovule (Fig. 8D).

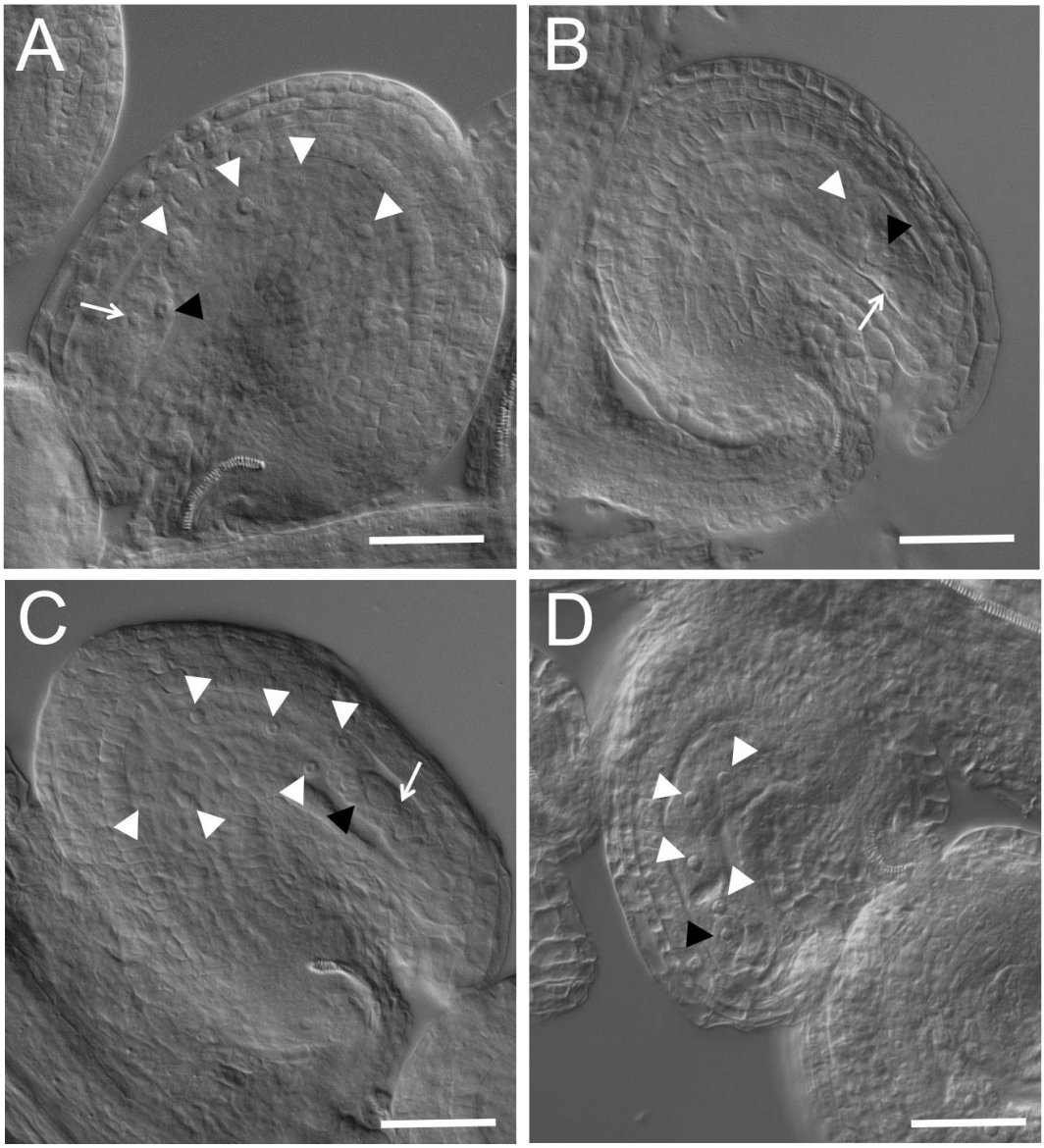

Figure 8 – Cleared whole mounts of *jagger* (A-C) and wild-type (D) seeds.

A. *jagger* developing seed with four nuclei-endosperm (white arrowheads), a zygote (black arrowhead) and a persisting synergid (white arrow). **B.** *jagger* developing seed with two endosperm nuclei with a recently formed zygote (black arrowhead) and a persisting synergid (white arrow). **C.** *jagger* developing seed with 6 visible endosperm nuclei a developing zygote (black arrowhead) and a persisting synergid (white arrow). D. Wild-type developing seed with a four-nucleate endosperm (white arrowheads) and a recently formed zygote (black arrowhead). Bars: 50 µm.

Ovules of *jagger2-/-* flowers at the same stage and pollinated with wild-type pollen were included in LR White resin and its sections observed under an optical microscope. The observations were in accordance with what was seen in the cleared ovules. A persisting synergid nucleus was visible near the micropylar region of the ovule, side by side with the developing embryo (Fig. 9A – C, E-F) and zygote (Fig. 9E).

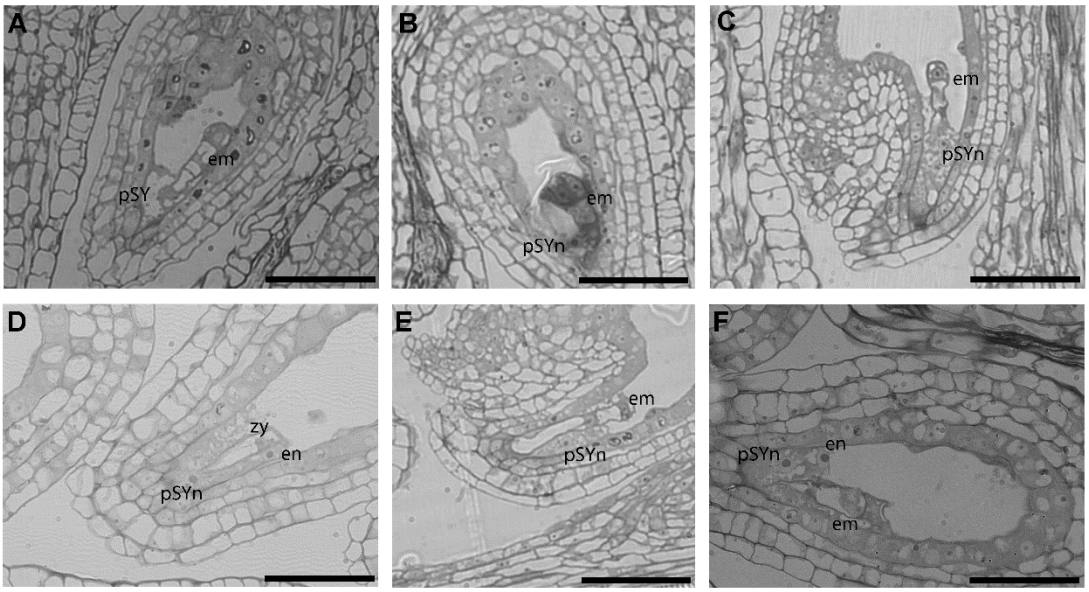

Figure 9 – LR-White cross sections of embedded *jagger* seeds.

A – C and E-F *jagger* seed with an already developing embryo and a persistent synergid next to it, near the micropylar region. **E.** jagger seed with a zygote and a persistent synergid nucleus near the micropylar end of the ovule. En – endosperm nucleus, em – embryo, pSY – persistent synergid, pSYn – persistent synergid nucleus, zy – zygote. Bars: 30 µm.

JAGGER possible interactors revealed by the Yeast Two Hybrid technique

To experimentally detect putative interactors for JAGGER we used the core domain of this protein as bait to screen a normalized Arabidopsis expression library. A whole plant

cDNA library was used as it was thought to be the best choice for identifying AGP interactors. The library was generated from pools of cDNAs obtained by mRNA extracted from several organs at different developmental stages including developing inflorescences, developing siliques, and mature flowers just before, and at anthesis. The library was normalized in order to achieve a better representation of low abundant messengers.

For the JAGGER library mating plates, 85 colonies grew on selective media. 50 among them were selected randomly for sequencing and they revealed 33 unique possible candidates. Y2H preliminary results revealed the list of possible interactors for JAGGER presented in Supplemental Table 2.

DISCUSSION

JAGGER is an AGP essential for polytubey block and might play a role in the programmed cell death of the persistent synergid

Polytubey is a term used to describe a circumstance when an embryo sac is targeted by more than one PT (Beale *et al.* 2012). Polytubey, if occurring, might lead to the fusion of more than one sperm cell with a female gamete, a situation termed "polyspermy". To guarantee a successful seed development the male and the female gametophytes must hold mechanisms to limit the number of sperm cells delivered into the embryo sac to only one pair of sperm cells (Scott *et al*. 2008). But, concurrently, the same gametophytes must tightly control this mechanism to assure fertilization of both female gametes in cases of sperm fusion failure with the female gametes (Kasahara *et al.* 2012). In several female gametophytic mutants where the PT fails to arrest its growth and does not burst releasing the sperm cells inside the embryo sac, ovules are capable of attracting more than one PT, such as in *feronia*/*siréne*, e.g. (Rotman *et al.* 2003; Huck *et al.* 2003; Capron *et al.* 2008; Tsukamoto *et al.* 2010, Boisson-Dernier *et al.* 2008). Even when the sperm cells are released inside the embryo sac but their fusion with the female gametes fail, as in the *egg cell1* case (Sprunck *et al.* 2012; Rademacher and Sprunck, 2014), ovules are able to attract more than one PT. It was shown recently that for the female gametophyte to block multiple PTs from entering, it must ensure that double fertilization is successful, that is, that two sperm cells fuse with the egg and the central cell, giving rise, respectively, to the embryo and the nourishing endosperm (Beale *et al.* 2012). By using defective gamete fusion *hap2* (*gcs1*) (von Besser *et al.* 2006) and *duo1* sperm (Durbarry *et al.* 2005) Beale and colleagues (2012) demonstrated that an embryo sac is capable of receiving up to four PTs as necessary to guarantee the double fertilization and that when this is accomplished the attraction of more PTs is blocked. At the same time Kasahara (2012) and colleagues carried out a very similar study, also using the defective gamete fusion *hap2* (*gcs1*), *duo1* and *duo3-2* sperm to pollinate wild-type pistils. The results were similar, with the difference that in this latest study no more than two PTs were ever observed entering one wild-type embryo sac, and lead to similar conclusions.

In Angiosperms, the synergids play a fundamental role as the source of attractants for PT targeting into the embryo sac micropylar opening in different species such as *Torenia fournieri* (Higashiyama *et al.* 2001, Okuda *et al.* 2009), *Zea mays* (Márton *et al.* 2005) and *Arabidopsis thaliana* (Kasahara *et al.* 2005, Takeuchi and Higashiyama, 2012).

Nonetheless, other studies highlighted the importance of the other female gametophytic cells in PT interactions with the female gametophyte. Chen *et al.* (2007) showed that the central cell was also essential for PT guidance. Scott *et al.* (2008) showed evidences for a polyspermy block by the egg cell but not the central cell in Arabidopsis. It is now known that both, the egg and the central cell, have the ability to control, independently, the attraction of multiple PTs: the polytubey situations (Maruyama *et al.* 2013). Several FIS (Fertilization Independent Seed) class mutants such as *medea/fis1* (Grossniklaus *et al.* 1998; Kinoshita *et al.* 1999; Kiyosue *et al.* 1999; Luo *et al.* 1999), *fis2* (Chaudhury *et al*. 1997; Luo *et al.* 1999) and *fertilization independent endosperm* (Ohad *et al.* 1996; Ohad *et al.* 1999) display the polytubey phenotype, as shown by Maruyama *et al.* (2013). All these mutants belong to a class of genes coding for proteins that belong to the Polycomb Repressive Complex 2 (PRC2), a chromatin-remodeling factor responsible for gene silencing, and are shown to be involved in the blockage of polytubey by the central cell. The study of ethylene insensitive transcription factors mutants responsible for the activation of the ethylene pathway, *ein3* and *ein2*, showed that ethylene is essential for the polytubey blockage (Volz *et al.* 2013). Ethylene acts by triggering a signaling cascade that will lead to the death of the persistent synergid, blocking the production of PT attractants.

In these study two independent mutant alleles were studied, *jagger1* and *jagger2*, and both presented the same defects in PT attraction, the polytubey phenotype. *jagger* phenotypic analyses revealed no impact on the maturation of the female gametophyte development, being all the cell types correctly specified, indicating that mutant embryo sacs reached maturity and are ready to be fertilized by the PTs. In *jagger,* fertilization occurs normally, as revealed by the crosses with the sperm cell marker line and by the correct development of embryo end endosperm in the developing seeds. The first PT arrives into one of the synergids bursting and releasing a pair of sperm cells, which fuse normally with the egg and the central cell. In every case where a second PT was attracted to the embryo sac, the pair of sperm cells is observed near the micropyle region or inside the already developing endosperm. Hence, this is one of the rare cases of a mutant where fertilization proceeds normally, and still, one more PT is attracted into the embryo sac, alike the ethylene mutants (Volz *et al.* 2013).

In *jagger* mutants no more than two PTs entering one embryo sac were ever observed, as in the case of Kasahara *et al.* (2012). This is most probably related to the presence of only two synergids in each Arabidopsis ovule. Once one synergid degenerates after the arrival of the first PT, the second persisting synergid will be the

source of PT attractants, targeting the extra PT. After invasion of this persisting synergid by a second PT it will automatically degenerate, ceasing the production of more PT attractants. This is shown by the presence of the persistent synergid long after the fertilization occurs. It is controversial in the literature the timing of PCD of this second synergid. According to Schneitz *et al.* (1995), "the synergids disappear during the second and third endosperm nuclear division". In *jagger* seeds, the persisting synergid is observed still in these developmental phases and even after, during the elongation of the zygote and the development of the embryo proper.

In conclusion, although this phenotype is not fully penetrant in *jagger*, its presence in both mutant alleles shows that JAGGER is essential for polytubey block in Arabidopsis, acting after double fertilization. It is involved in the PCD of the persisting synergid, and consequently in the cessation of PT attractant production, consequently avoiding polytubey. JAGGER belongs to a family of highly glycosylated proteins (Seifert and Roberts 2007) so, if JAGGER itself or it sugar contents are the reason of this phenotype by activating a blockage system to polytubey, either through the entrance of a JAGGER-primed PT into the embryo sac or by acting after fertilization, in the ethylene blocking pathway, further analyses are needed. Yet, the polytubey phenotype in not fully penetrant in both single mutants analyzed, *jagger1* and *jagger2*, suggesting that other genes might be necessary for this function. Given the large number of genes included in the AGP family, some gene redundancy is expected (Kafri *et al.* 2009), as already evidenced for *AGP6* and *AGP11* (Coimbra *et al.* 2010). So in this system, the analysis of multiple mutants of related AGPs will also be important.

JAGGER possible interactors reveal similarities between pollen tube growth and fungal invasion

To identify putative JAGGER interactors, yeast two-hybrid screenings have been set up using a normalized *Arabidopsis thaliana* total plant cDNA library. Interesting possible interactors for JAGGER were revealed by this procedure, most of them involved in plants' fungal invasion (Table 2). PT growth through the pistil tissues resembles, in many ways, the invasion of plant tissues by fungal hyphae and oomycetes. In both situations there must be recognition by specific signaling molecules and receptors in order to allow or not the growth of the PT or the pathogen. Plant tissues must allow or protect themselves from this external growing cells, identifying them as beneficial or pathogenic. Some studies have already speculated about the similarities between these two processes (Dresselhaus and Márton, 2009; Nibau and Cheung, 2011) and others have shown the involvement of

the same molecules in both processes such as the case of FERONIA (Kessler *et al.* 2010) and the tomato defensing DEF2 (Stotz *et al.* 2009).

Putative AGP4 interactor	Function
AP2C1 (Arabidopsis Ser/Thr Phosphatase of type 2C)	Acts a MAPK (Mitogen Activated Protein Kinase) phosphatase.
PMR6 (Powdery Mildew Resistant 6)	Pectate lyase-like protein involved in pectin degradation in the plant cell wall.
ATSOT16 (desulfoglucosinolate sulfotransferase)	Involved in the final step of glucosinolate core structure biosynthesis.
RIPK (RPM – Induced Protein Kinase)	Phosphorylates the host target RIN4, leading to the activation of a plant innate immune receptor RPM1.
At3g19390 (cysteine protease)	Cysteine-type endopeptidase activity, involved in proteolysis.
AtMYC2 (bHLH Leucine zipper transcription factor)	Binds to an extended G-Box promoter motif and interacts with Jasmonate ZIM-domain proteins.
BGLU18 (Beta Glucosidase 18)	Member of glycosyl hydrolase family 1, located in inducible ER bodies formed after wounding.
SSRP1 (Structure Specific Recognition Protein1)	High Mobility Group domain-containing component of FACT (Facilitates Chromatin Transcription) histone chaperone, required for DNA demethylation.

Table 2 – Set of AGP4 putative interactors involved in pathogen responses in Arabidopsis.
(Information from TAIR, The Arabidopsis Information Resource [https://www.arabidopsis.org/index.jsp]).

AP2C1 (Arabidopsis Ser/Thr Phosphatase of type 2C), acts as a MAPK (Mitogen Activated Protein Kinase) phosphatase that negatively regulates MPK4 and MPK6. AP2C1 overexpression results in reduced MPK6 and MPK4 activities, reduced ethylene production and a consequently increased susceptibility to plant infection by *Botrytis cinerea* (Schweighofer *et al.* 2007). It is interesting to relate this possible interaction with the ethylene function in blocking polytubey and promoting synergid cell death. Defense mechanisms activated by ethylene may result in a hypersensitive response activation and PCD induction, concomitant with reactive oxygen species (ROS) accumulation (León and Montesano, 2013). ROS accumulation was observed after pollination and right before fertilization in the synergids (Martin *et al.* 2013).

PMR6 encodes a GPI anchored pectate lyase-like protein responsible for pectin

degradation in the plant cell wall and *pmr6* plants have increased resistance to powdery mildew infection, and high levels of pectin accumulated in cell walls near infection sites (Vogel *et al.* 2002). The resistance to infection probably comes from the inaccessibility of the fungus to nutrients from the host cell. Unlike other resistant mutants, *pmr6* is independent of any host defense mechanism activation. According to eFp Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter *et al*. 2007) PMR6 is also highly expressed in pistil tissues, although no function as been assigned to it related to sexual reproduction. It might as well be important for loosening the cell walls through the PT growth pathway to facilitate targeting to the ovule.

ATSOT16 is a desulfoglucosinolate sulfotransferase important for glucosinolate synthesis. It is activated by ethylene after wounding, moreover glucosinolates play a role counteracting pathogen defense (Piotrowski *et al.* 2004). RIPK kinase is another possible interactor for JAGGER that associates with and modifies an effector-targeted protein complex to initiate host immunity, although very little is known about this kinase targets (Liu *et al.* 2011). Interestingly a cysteine protease, At3g19390, was also found as a possible interactor with JAGGER, this is known to act during PCD of endothelium cells during seed development (Ondzighi *et al.* 2008). AtMYC2, a basic helix-loop-helix Leucine zipper transcription factor, which is a positive regulator of ABA signaling, is also involved in activating jasmonic acid ethylene responsive pathways. It modulates defense and stress responsive gene expression in response to biotic stresses (Anderson *et al.* 2004). A BGLU18 (beta glucosidase 18) that is localized in inducible ER bodies seem to have a role in plant defense systems, by responding to wounding by pathogens and is related to ethylene signal transduction pathways (Ogasawara *et al.* 2015). One of the most interesting candidates for interaction with JAGGER is SSRP1 (STRUCTURE SPECIFIC RECOGNITION PROTEIN1), an HMG (High Mobility Group) domain-containing component of FACT (Facilitates Chromatin Transcription) histone chaperone, that is required for DNA demethylation and for activation and repression of parentally imprinted genes in the central cell (Ikeda *et al.* 2011; Ikeda, 2012). This may be related to the FIS-PRC2 complex regulating polytubey blockage.

Analysis of these Y2H preliminary results indicate that JAGGER may be involved in the intricate signaling pathway that leads to PT growth targeting to the embryo sac in a way similar to what happens in a fungal invasion. An AGP was already shown to be involved in fungal invasion in *Nicotiana alata*, NaAGP4 (Gilson *et al.* 2001), a homologue of the tomato LeAGP1 that is rapidly suppressed by tissue wounding and by pathogen infection (Gao *et al.* 1999). Curiously, both AGPs are enriched in the pistil transmitting

tissues, like AtAGP4, studied in this work. AGPs were also shown to be involved in a process called “pollen mimicry” during infection of blueberry flowers (Ngugi and Scherm, 2004, 2006), where the fungus *Monilinia vaccinii-corymbosi* infects blueberry pistils by mimicking PT growth. Epitopes of AGPs were identified not only in blueberry flower pistil transmitting tissue but also in the fungal conidia and hyphae. So, the question that arises is: who is mimicking whom? Some researchers postulate that the evolution of flowering plants mode of reproduction mimics the mode of fungal invasion in plants and takes advantage of the many genes involved in defense already present in ancient water-plants (Dresselhaus and Márton, 2009). But “here” we have fungi exploiting a perfect road for pollination and seed production to avoid natural defense barriers from plants, such as in the blueberry flowers. This is a stimulating field of research, from an evolutionary point of view, with many unexplored molecular mechanisms waiting to be known.

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CHAPTER 5

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Taking into account the possible roles of AGPs either as signalling or nutrient molecules and the differential presence of AGP carbohydrate epitopes in space and time during the development of reproductive tissues, male and female, it is tantalising to speculate about the possible functions of AGPs in sexual plant reproduction. AGPs are strong candidates to be major players in the different steps of interaction between the pollen grain, the pollen tube and the pistil tissues:

1. AGPs might be important for PG recognition, adhesion and germination, once it is in contact with the stigmatic cells, and for PT growth initiation;

2. AGPs have all the potential characteristics to function as guiding, support and nutrient providing molecules for PT growth through the transmitting tract or mucilage of hollow pistils, along the style and the ovary;

3. AGPs are also present in the ovules and funiculus, being excellent candidates as attracting molecules for this final stage of pollen tube micropylar guidance;

4. AGP presence in some embryo sac cells suggests the possible involvement of these molecules in the control of sperm cell-egg cell fusion as well as in multiple pollen tube blockage (Pereira *et al.* 2015, submitted).

Two main features became clearer with the research undertaken during all these years. One is the increasing supporting information on the importance of the carbohydrate moiety of AGPs for its functions. As Wu *et al.* (2000) had already suggested, the different patterns of glycosylation of AGPs might be extremely important in regulating their biological activity. A striking example are the TTS proteins from tobacco, that when deglycosylated, were unable to stimulate PT growth and attraction in vitro (Cheung *et al.* 1995). A very elegant study made by Higashiyama (2014) highlighted the importance of AGP sugars in reproduction, by discovering a new key molecule in PT guidance: the AMOR glycan. Secondly, the similarities between different amino acid sequences of AGPs may be relevant only because it might determine the post-translational patterns of glycosylation of these proteins, but they might be irrelevant to define AGP functions per se, meaning that AGPs, even if closely related at the amino acid level, might play unique and different functions in different processes, a hypothesis already suggested by de Graaf *et al.* (2003), Yang et al. (2007) and Pereira *et al.* (2014), and which is in agreement with

some particularly important recent studies in reproduction, that suggest the involvement of polysaccharides as putative ligands to FERONIA, a receptor-like kinase (Escobar-Restrepo *et al.* 2007; Lindner *et al.* 2012), essential for the success of double fertilization. Although their exact nature is yet to be determined, these putative ligands could be AGPs sugar components.

While a huge amount of information is currently available on AGP distribution throughout sexual reproductive tissues in many species, information regarding its functions in this process is still scarce. We think that a new era for the study of AGP functions in sexual reproduction is just beginning and that, maybe sooner than expected, a lot of new information will clarify not only AGP functions but also their mode of action.

AGPs presence across all different species of Angiosperms, basal Angiosperms and even in Gymnosperms sexual reproductive tissues is remarkable. The facts that these genes are conserved across all these plant species may indicate their importance for plants survival. Many functions have been implied for these proteins over the years in plant development (Majewska-Sawka and Nothnagel, 2000). When it comes to reproduction, only to AGP18 (Acosta-García and Vielle-Calzada, 2004; Demesa-Arévalo and Vielle-Calzada, 2013), AGP6 and AGP11 (Coimbra *et al.* 2009, 2010) specific functions were assigned. Regarding the plant model species *Arabidopsis thaliana*, information was available regarding the differential spatial and developmental presence of Arabinogalactan epitopes along the reproductive tissues (Coimbra *et al.* 2007).

The overall aim of this thesis was to gain further insights into the localization and function of specific AGPs along the several reproductive tissues, since the moment of pollen grain adhesion into the stigma until the release of the sperm cells inside the embryo sac. The main objectives were achieved successfully. As it is exhaustively depicted in Chapter 2, AGPs revealed to be important not only in Angiosperms reproduction, but also in a basal Angiosperm such as in Thrituria. AGP1, AGP9, AGP12, AGP15 and AGP23 cellular localizations were described in detail in Chapter 3 as well as their putative roles in the reproductive processes. One of the AGPs studied in more detail revealed to be fundamental for blocking the growth of supernumerary pollen tubes into one embryo sac, as it is described and discussed in more detail in Chapter 4. Since most of the Chapters compile in detail Discussions regarding each issue described, it was decided not to further discuss these subjects in this Chapter, not to become redundant.

There is a clear connection between each one of the Chapter, since the use of the most old techniques like monoclonal antibodies to study the presence of AGPs in the

basal Angiosperm Thrituria, through the detailed description of a group of AGPs pattern of expression in the model species reproductive tissues until the findings regarding the function of one important AGP, the AGP4 or JAGGER.

Some T-DNA insertion mutants of AGPs were analysed during the period of these PhD thesis for AGPs with available knock-out mutants: AGP1, AGP10, and AGP20. *agp1*, *agp10* and *agp20* studies showed no visible phenotype and therefore we decided not to include this data in the results. This was most probably due to the high probability of redundancy occurrence in this family of proteins, as already mentioned above in Discussion of Chapter 3 and 4.

Much more needs to be done in order to make more progresses in the study of these specific AGP. The next steps of its study will include the search for putative receptors for AGP4 using techniques such as the Yeast two Hybrid approach, as well as the RNA seq methodology in *agp4* and wild-type plants. We hope to get further information about the molecular network in which this glycoprotein may be acting and in this way to fully assign its molecular function, the most challenging project. It will be also essential to search for other genes that might be acting redundantly with JAGGER, and carry on with further analyses of double or even triple mutants, in order to try to get a mutant with a higher level of polytubey.

Regarding AGPs specific localization in plant reproductive tissues more studies must also be carried on regarding the other AGPs, despite the absent signals obtained in several microarray analyses. Countless times, genes are expressed in such a specific timing, location and in a relative small quantity, that the microarray techniques are not able to detect them. Even thou, these genes might as well be playing major roles in several steps of the reproductive process.

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APPENDIX 1

SUPPLEMENTAL MATERIAL FROM CHAPTER 3

Supplemental table 1 - Primer list for AGP promoter amplification and use in GUS/GFP fusions

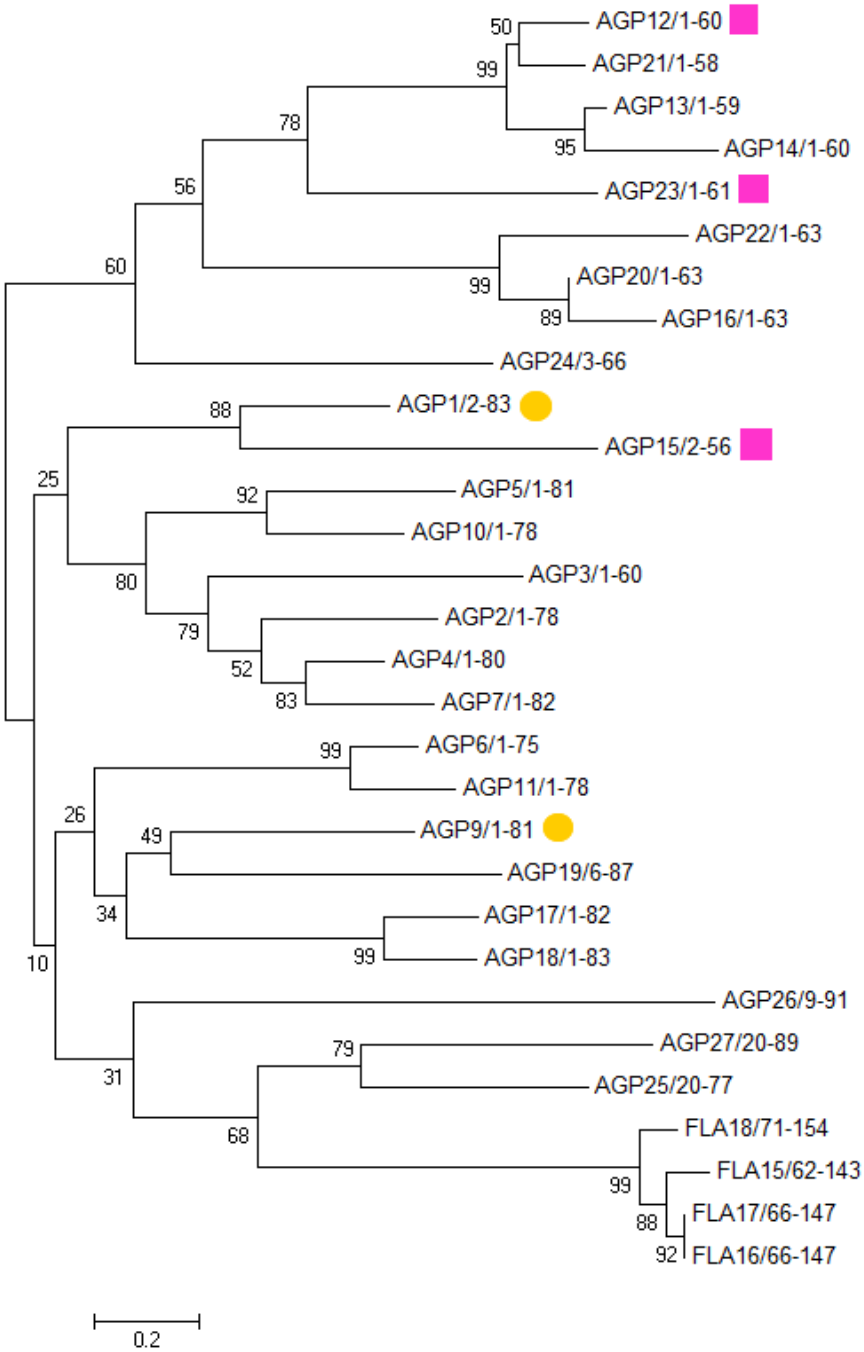
Name	Locus	Primers
AGP1	At5g64310	**F CACC**GTCATATTGACTCTGAGCCATAAACTC **R** CAAAAAAGAGAGAGATTCGAATTTAGC
AGP9	At2g14890	**F CACC**ATTGGCCACAGTTCACCTGC **R** TTTTGCTTTTGCTTTTTCTCTCTG
AGP12	At3g13520	**F CACC**GTTGGGGCCACATTTGTAGT **R** CTTCTAAGTGCAAAAGAGGAG
AGP15	At5g11740	**F CACC**CCATTTTCTTTGATTGTAGCAAGTTAG **R** TTCAAAGATTTTGTTGTGAGAGATAAAG
AGP23	At3g57690	**F CACC**CATCGTGAATATTTATAGGACAAGTTTATG **R** GAGACCTGAAAGGCTTTTCTTTTC

Supplemental table 2 - Primer list for the Real Time RT-PCR experiment with the selected AGPs

Name	Locus	Primers
AGP1-RT	At5g64310	**F** CAAAAACACTCCCAAACCAAA **R** GGTGGTGAAGCAGAGGATTC
AGP4-RT	At5g10430	**F** TCGCCACTTCAGCACTCGCTC **R** CGGGAGCACTGCTTGGGCTC
AGP7-RT	At5g65390	**F** GAACTAGCCCCGACACCTTC **R** ACAAGTGAACCGACGACGAA
AGP9-RT	At2g14890	**F** ATCTGTATCGTCCTCATCG **R** ATGTTGTGACTGGTGGTG
AGP10-RT	At4g09030	**F** CTGCTCAACCTCCAAGAACC **R** CAAATCCGGCTAAAGATCCA
AGP12-RT	At3g13520	**F** ACAACTCATCATTCGCACCA **R** GCATCGGAAGTAGGACTTGG
AGP15-RT	At5g11740	**F** CTCAGCGTCTTTCGTGTCAG **R** GCTTCGGAATACACACGAGAC
AGP16-RT	At2g46330	**F** TCATCATTTTCGTCGGATCA **R** ACCACCATTAGCAAATACGC
AGP23-RT	At3g57690	**F** AATGGAGATGAAGAAGATTG **R** TGCAAGTAGTAGCTGAAG
AGP25-RT	At5g18690	**F** ACGAACGTTTTGGTTTTCACG **R** TCAAGAGGGGCAAACACGAC
AGP26-RT	At2g47930	**F** CAAGCGAAATGTCTCCTTCC **R** TGCTGCTTACTCGCTGTTTC
RUB1	At4g36800	**RT_147** CTGTTCACGGAACCCAATTC **RT_148** TGTCGGTCAGACCTTTTTCC
ACT8	At1g49240	**RT_861** CTCAGGTATTGCAGACCGTATGAG **RT_862** CAGAGTATGATGAAGCAGGTCCAG

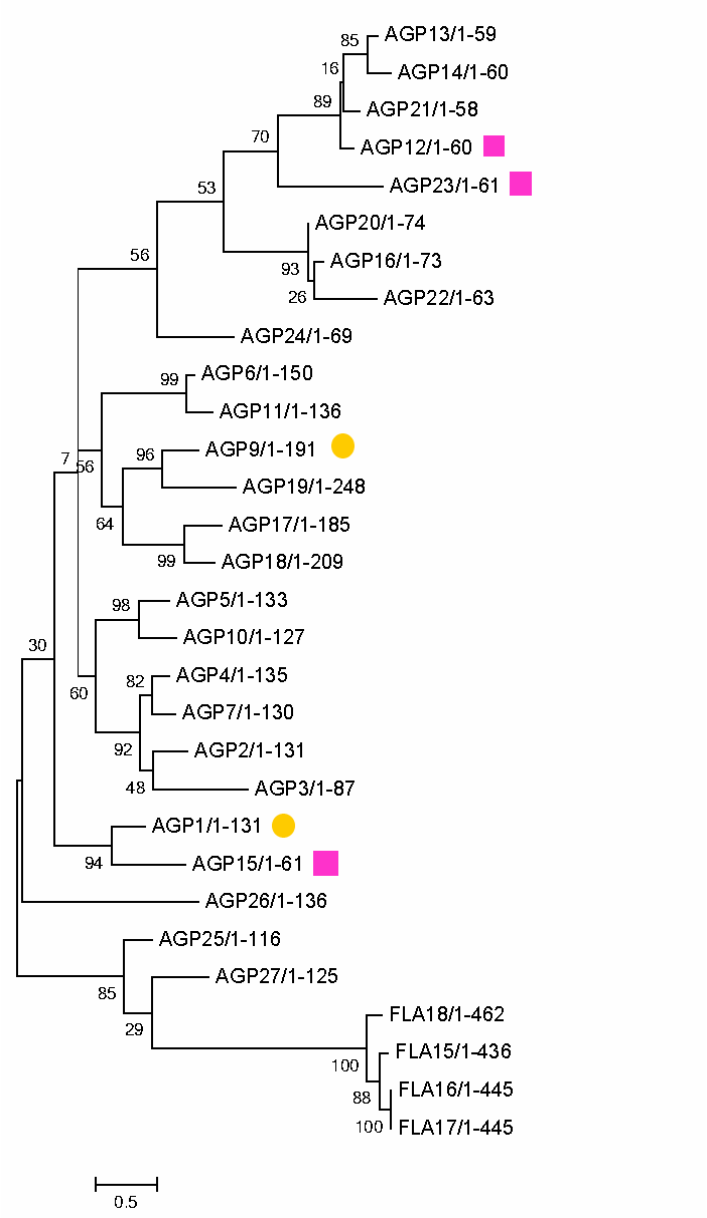
Supplemental figure 1 – Phylogenetic analysis of the AGP family in A. thaliana.

To generate the phylogenetic tree for AGPs, the 3 blocks of most conserved amino acid sequences of AGPs coding sequences were aligned using Clustal W and manually edited using Jalview to reduce gaps. Neighbor-joining (NJ) tree was generated using the MEGA4 program. AGPs selected for further analysis are indicated by a yellow circle (classical AGPs) and a violet square (AG peptides).



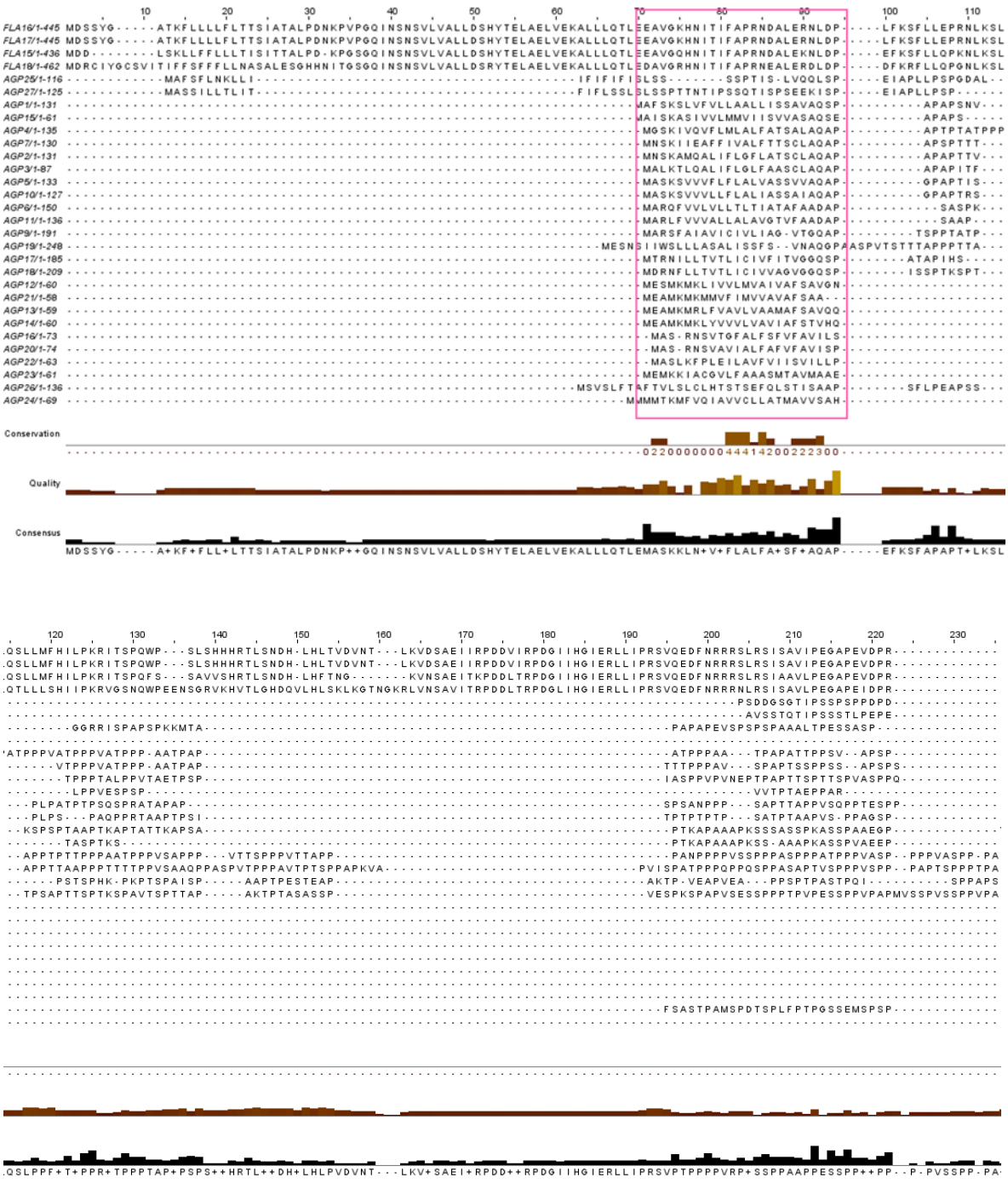
Supplemental figure 2 – Phylogenetic analysis of the AGP family in A. thaliana.

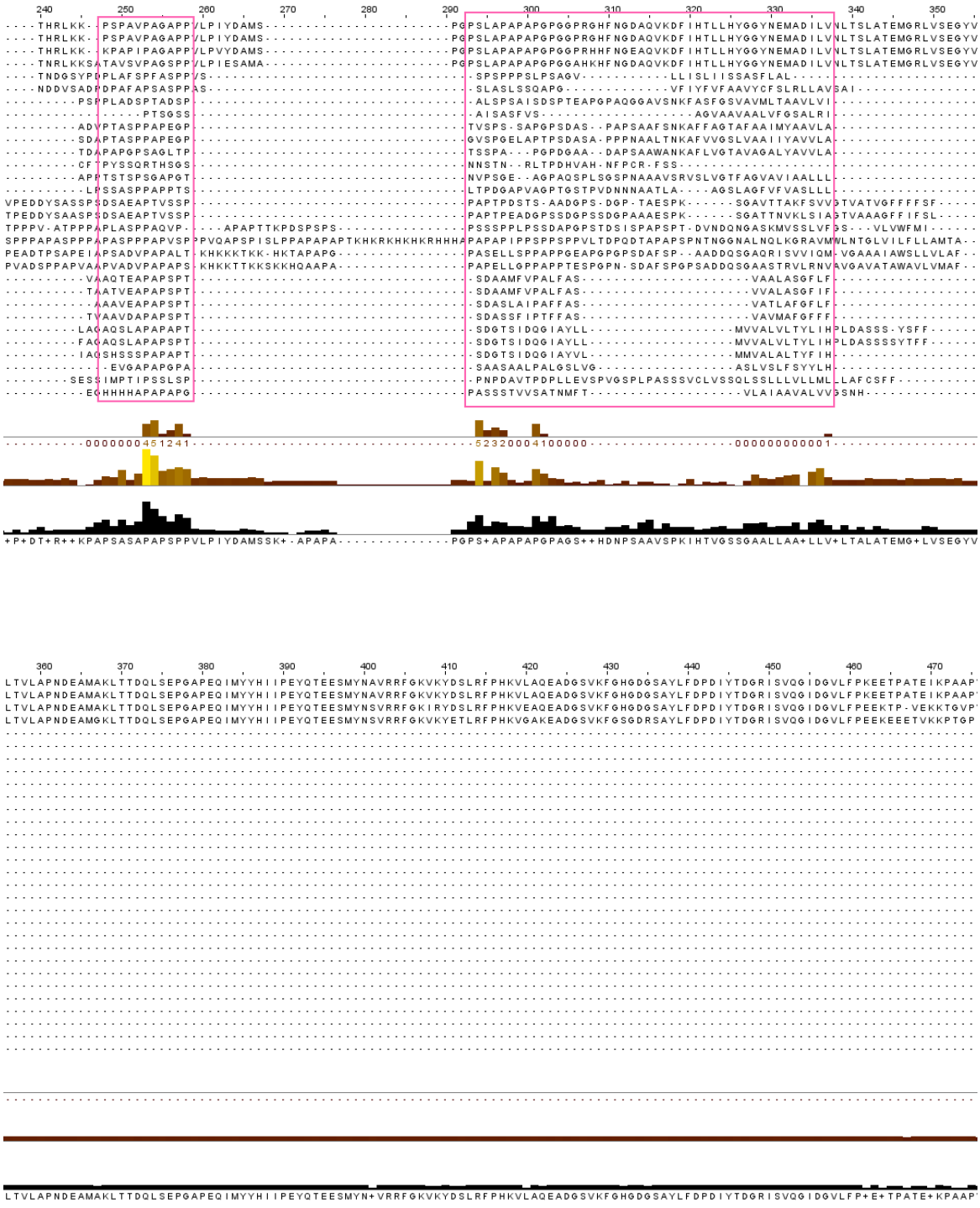
To generate the phylogenetic tree for AGPs, the 3 blocks of most conserved amino acid sequences of AGPs coding sequences were aligned using Clustal W and manually edited using Jalview to reduce gaps. Maximum Parsimony tree was generated using the MEGA4 program. AGPs selected for further analysis are indicated by a yellow circle (classical AGPs) and a violet square (AG peptides).

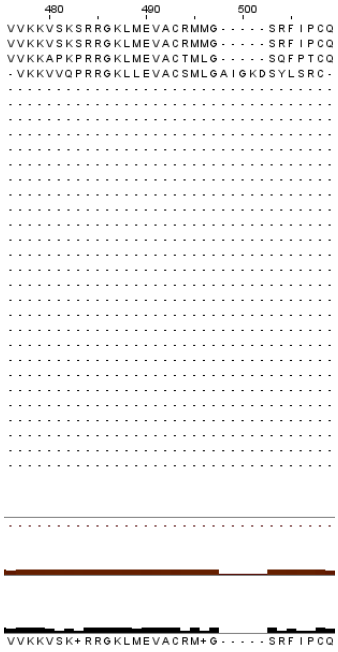


Supplemental figure 3 – The amino acid sequences of AGPs coding sequences aligned using Clustal W and viewed using Jalview.

The 3 conserved blocks are highlighted by the pink rectangles.






480
490
500
VVKKVSKSRRGKLMEVACRMMG-----SRFIPCQ
VVKKVSKSRRGKLMEVACRMMG-----SRFIPCQ
VVKKAPKPRRGKLMEVACTMLG-----SQFPTCQ
-VKKVVQPRRGKLLEVACSMLGAIGKDSYLSRC-
VVKKVSK+RRGKLMEVACRM+G-----SRFIPCQ

APPENDIX 2

SUPPLEMENTAL MATERIAL FROM CHAPTER 4

Supplemental table 1 - Primer list used in the different experiments.

Primers used for jagger1 and jagger 2 genotyping	
LP-GK-134A10	TGTCTCCCCACATTTGCCAT
RP-GK-134A10	ACAACCATATGAAGCCCTTCC
DS34	CCGTCCCGCAAGTTAAATATG
08409	ATATTGACCATCATACTCATTGC
Primers used for obtaining JAGGER different constructs	
AtP_4390	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTTTTTCCATTGTCTCAATTTG*
AtP_4391	GGGGACCACTTTGTACAAGAAAGCTGGGTATGCTTCTTCTTCTTTTGGTGTT*
AtP_4486	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGGTTCCAAGATTGTCCAAG*
AtP_4487	GGGGACAAGTTTGTACAAAAAAGCAGGCTGAATATTTATAGGACAAGTTTATG*
	*attB1 and attB2 adaptor sequences for recombination are underlined.
Primers used in Real Time RT-PCR experiments	
RUB1-fw	CTGTTCACGGAACCCAATTC
RUB1-rv	TGTCGGTCAGACCTTTTTCC
ACT8-fw	CTCAGGTATTGCAGACCGTATGAG
ACT8-rv	CAGAGTATGATGAAGCAGGTCCAG
AGP4-RT-fw	TCGCCACTTCAGCACTCGCTC
AGP4-RT-rv	CGGGAGCACTGCTTGGGCTC
AGP7-RT-fw	GAACTAGCCCCGACACCTTC
AGP7-RT-rv	ACAAGTGAACCGACGACGAA

Primers used to obtain in situ hybridization probes

AGP4insitufw	GGCTCTATTCGCCACTTCAG
AGP4insitufwT7	TAATACGACTCACTATAGGGGGCTCTATTCGCCACTTCAG*
AGP4insiturv	AACGGCGGCGTACATAATAG
AGP4insiturvT7	TAATACGACTCACTATAGGGAACGGCGGCGTACATAATAG*

*T7 adaptors underlined.

Primers used to amplify AGP4 coding sequence for Y2H

AGP4_fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGCAAGCCCCTGCTCCTACTCCCAC
AGP4_rv	GGGGACCACTTTGTACAAGAAAGCTGGTCCTAGGGGCAGGGGATGCATCTGAAG

Supplemental table 2 - Interactors of JAGGER identified by Y2H library screening assays.

	AGI locus	Gene description	Subcellular localization
JAGGER	At5g42020	Luminal binding protein (BiP2) involved in polar nuclei fusion during proliferation of endosperm nuclei.	ER lumen
	At5g65640	Beta HLH protein 93 (bHLH093); DNA binding, sequence-specific DNA binding transcription factor activity.	nucleus
	At5g19330	ARIA, ARM REPEAT PROTEIN INTERACTING WITH ABF2 - Encodes an armadillo repeat protein involved in the ABA response. The protein interacts with a transcription factor, ABF2, which controls ABA-dependent gene expression via the G-box-type ABA-responsive elements.	cytoplasm, nucleus, p. membrane
	At3g01090	AKIN10 - Encodes a SNF1-related protein kinase that physically interacts with SCF subunit SKP1/ASK1 and 20S proteosome subunit PAD1. It can also interact with PRL1 DWD-containing protein. Based on in vitro degradation assays and cul4cs and prl1 mutants, there is evidence that AKIN10 is degraded in a proteasome-dependent manner, and that this depends on a CUL4-PRL1 E3 ligase.	nuclear ubiquitin ligase complex, nucleus
	At1g03140	Splicing factor Prp18 family protein.	nucleus, spliceosome complex
	At5g61410	D-Ribulose-5-Phosphate-3-Epimerase, EMB2728, EMBRYO DEFECTIVE 2728, RPE.	apoplast, chloroplast, chloroplast envelope, chloroplast stroma, thylakoid
	AtCg00470	ATP Synthase Epsilon Chain, ATPE.	chloroplast, chloroplast envelope, chloroplast thylakoid, chloroplast thylakoid membrane, proton-transporting ATP synthase complex, catalytic core F(1),thylakoid
	At2g45820	Remorin family protein.	plasma membrane
	At2g30020	Encodes AP2C1. Belongs to the clade B of the PP2C-superfamily. Acts as a MAPK phosphatase that negatively regulates MPK4 and MPK6.	plastid, protein serine/threonine phosphatase complex

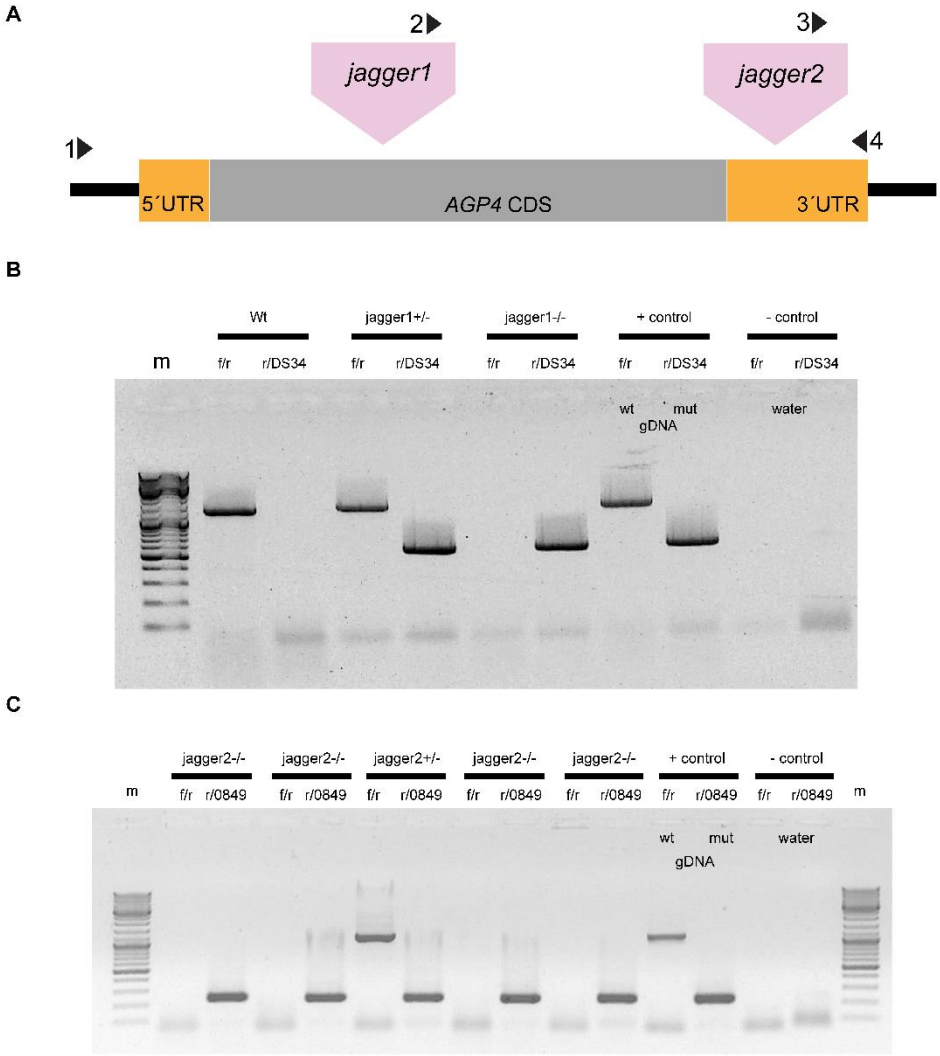
AGI locus	Gene description	Subcellular localization
At3g54920	PMR6, Powdery Mildew Resistant 6 – encodes a pectate lyase-like protein.	anchored to membrane
At3g08850	RAPTOR1 - Encodes one of two Arabidopsis RAPTOR/KOG1 homologs. RAPTOR proteins are binding partners of the target of rapamycin kinase that is present in all eukaryotes and play a central role in the stimulation of cell growth and metabolism in response to nutrients. Mutants show embryo lethal phenotype which occurs at pre-globular stage. May interact with TOR kinase in a rapamycin like signaling pathway. Interacts with TOR and S6K1 in vivo. Overexpression of RAPTOR1 rendered the S6K1 osmotic stress insensitive.	CUL4-RING ubiquitin ligase complex
At1g74100	ATSOT16, ATST5A - encodes a desulfoglucosinolate sulfotransferase, involved in the final step of glucosinolate core structure biosynthesis. Expression was induced by wounding, jasmonate and ethylene stimulates.	cytoplasm
At4g18890	BEH3, BES1/BZR1 HOMOLOG 3 – involved in hyperosmotic salinity response, regulation of transcription, DNA-dependent, response to abscisic acid stimulus, response to water deprivation.	nucleus
At3g55440	ATCTIMC, Cytosolic Isoform Triose Phosphate Isomerase, CYTOTPI, TPI, Triosephosphate Isomerase.	apoplast, cell wall, chloroplast, chloroplast stroma, cytoplasm, cytosol, mitochondrion, plasma membrane, plasmodesma, vacuolar membrane,vacuole
At3g51520	ATDGAT2, DGAT2, Diacylglycerol Aacyltransferase 2	chloroplast
At3g01910	AT-SO, ATSO, SOX, Sulfite Oxidase	chloroplast, mitochondrion, peroxisome
At3g19390	Granulin repeat cysteine protease family protein; Has cysteine-type endopeptidase activity; Involved in proteolysis.	extracellular region
At2g05940	RIPK, RPM1-INDUCED PROTEIN KINASE - Encodes a receptor-like cytoplasmic kinase that phosphorylates the host target RIN4, leading to the activation of a plant innate immune receptor RPM1.	plasma membrane

AGI locus	Gene description	Subcellular localization
At1g52400	*A. thaliana* β-Glucosidase 1, ATBG1 - encodes a member of glycosyl hydrolase family 1, located in inducible ER bodies which were formed after wounding, required in inducible ER body formation.	ER body, chloroplast, endoplasmic reticulum, extracellular region, nucleus, peroxisome, plasmodesma, vacuole
At5g55070	Dihydrolipoamide succinyltransferase. Functions in zinc ion binding, acyltransferase activity; Involved in response to oxidative stress.	cytosolic ribosome, mitochondrion, oxoglutarate dehydrogenase complex
At2g43680	IQ-DOMAIN 14, IQD14 – Functions in calmodulin binding.	nucleus, plasma membrane
At3g12610	DNA-DAMAGE REPAIR/TOLERATION 100, DRT100 - Plays role in DNA-damage repair/toleration. Partially complements RecA-phenotypes.	chloroplast, plasma membrane
At4g30550	Class I glutamine amidotransferase-like superfamily protein.	cytoplasm, cytosol
At2g10940	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein; Functions in lipid binding and it's involved in lipid transport.	apoplast, chloroplast, chloroplast thylakoid membrane, membrane, plasmodesma
At3g26650	GAPA, Glyceraldehyde 3-Phosphate Dehydrogenase A Subunit – Encodes one of the two subunits forming the photosynthetic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and as such a constituent of the supramolecular complex with phosphoribulokinase (PRK) thought to be linked by a small peptide encoded by CP12-2.	apoplast, chloroplast, chloroplast envelope, chloroplast stroma, chloroplast thylakoid membrane, membrane
At5g64350	Arabidopsis Thaliana FK506-Binding protein 12, ATFKBP12, FK506-Binding Protein 12, FKBP12, FKP12 - Encodes FK506-binding protein 12 (FKBP12 or FKP12). FKP12 overexpression dramatically enhances rapamycin sensitivity, whereas rapamycin inhibition is relieved in transgenic plants deficient in FKP12.	chloroplast thylakoid lumen, cytoplasm, cytosol, membrane
At1g55310	AT-SCL33, SC35-Like Splicing Factor 33, SCL33, SR33 - Encodes a SR spliceosome protein that is localized to nuclear specks, interacts with SR45 and the U1-70K protein of the U1 snRNP, has sequence similar to human SC35 protein. Barta et al (2010) have proposed a nomenclature for Serine/Arginine-Rich Protein Splicing Factors (SR proteins).	interchromatin granule, nuclear speck, nucleolus, nucleus, plasma membrane

AGI locus	Gene description	Subcellular localization
At1g01320	Tetratricopeptide repeat (TPR)-like superfamily protein	cytoplasm
At1g32640	ATMYC2, JAI1, Jasmonate Insensitive 1, JIN1, MYC2, RD22BP1, ZBF1 -Encodes a MYC-related transcriptional activator with a typical DNA binding domain of a basic helix-loop-helix leucine zipper motif. Binds to an extended G-Box promoter motif and interacts with Jasmonate ZIM-domain proteins. Its transcription is induced by dehydration stress and ABA treatment. Positive regulator of lateral root formation. Regulates diverse JA-dependent functions. Negatively regulates Trp metabolism and biosynthesis of Trp-derived secondary metabolites. Positively regulates flavonoid biosynthesis, resistance to insects, and response to oxidative stress. Regulates other transcription factors, and negatively regulates its own expression.	nucleus
At4g17330	ATG2484-1, G2484-1, G2484-1 Protein - involved in cytokinesis by cell plate formation, gravitropism, microtubule cytoskeleton organization.	nucleus, plasmodesma
At5g55070	Dihydrolipoamide succinyltransferase; Functions in zinc ion binding, acyltransferase activity; Involved in response to oxidative stress, metabolic process.	cytosolic ribosome, mitochondrion, oxoglutarate dehydrogenase complex
At3g28730	ATHMG, High Mobility Group, HMG, NFD, Nucleosome/Chromatin Assembly Factor D, SSRP1 - encodes a component of the FAcilitates Chromatin Transcription (FACT) complex, SSRP1. Along with STP16 binds to the promoter of FLC.	nuclear euchromatin, nucleus

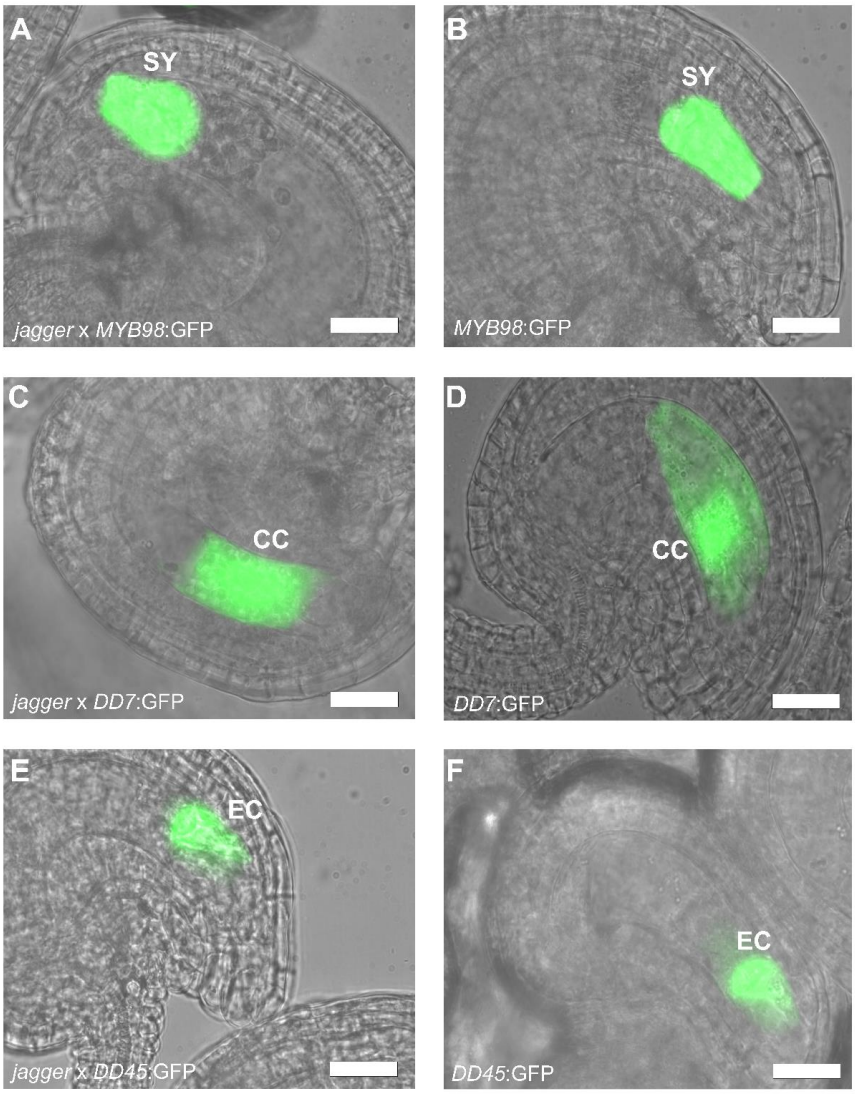
Supplemental figure 1 – Identification of JAGGER T-DNA insertions.

A. Positions of T-DNA and Ds insertions in JAGGER are indicated by inverted triangles, the grey box represents the exon, the yellow boxes represent the untranslated regions and the black lines represent non-coding regions. *jagger1* is the RIKEN pst 20518 line and *jagger2* is the GABI-Kat 134A10 line. The arrows represent the primers used for genotyping the plants (1: LP-GK-134A10; 2: DS34; 3: 0849; 4: RP-GK-134A10). **B.** Amplification of the Ds transposon insert in homozygous jagger2-/-, jagger2+/- mutant lines and wild-type. **C.** Amplification of the T-DNA insert in homozygous jagger2-/-, jagger2+/- mutant lines and wild-type. f: LP-GK-134A10; r: wt: wild-type; mut: mutant; m: DNA ladder.



Supplemental figure 2 – *jagger*-/- crossed with synergids, central cell and egg cell GFP marker lines.

A. *jagger*-/- crossed with the synergid marker line *MYB98*:GFP. The synergids express this gene correctly. **B.** Wild-Type ovules expressing the construct *MYB98*:GFP normally. The green GFP signal is present in the synergids as expected. **C.** *jagger*-/- crossed with the central cell marker line *DD7*:GFP. The central cell express the gene properly. **D.** Wild-Type ovules expressing the construct *DD7*:GFP normally. The green GFP signal is present in the central cell as expected. **E.** *jagger*-/- crossed with the egg cell marker line *DD45*:GFP. The egg cell express the gene as it should be. **F.** Wild-Type ovules expressing the construct *DD45*:GFP normally. The green GFP signal is present in the egg cell as expected. CC – central cell; EC – Egg cell; SY – synergids. Bars: 20 µm.



Supplemental figure 3 – Relative expression of JAGGER in wild-type and 35s$_{pro}$:JAGGER mutant flowers.

Plants 1, 2 and 3 were the only 3 independent lines containing the 35s$_{pro}$:*JAGGER* constructs that survived the BASTA treatment. From these plants, only plants 1 and 2 were overexpressing *JAGGER* relative to the Wild-type plants. The relative gene expression was measured using stably expressed reference genes (At4g27960 and At1g06780) in three biological samples with similar results. The data correspond to the ratio of the expression in wild-type or 35s:*JAGGER* lines compared to the wild-type and are the mean ± sd of three technical replicates of a biological sample.